REMARKS

Amendments to the Specification:

Applicants have herein amended the specification to recite the original priority claim of this application. This is the same priority claim as that shown on the filing receipt received in connection with this application. A copy of the filing receipt is attached hereto as Appendix 1 for the Examiner's convenience. No new matter is added by this amendment.

In support of this amendment, Applicants respectfully direct the Examiner's attention to the United States Patent and Trademark Office Official Gazette Notice of 18 March 2003 (enclosed as Appendix 2 for the convenience of the Examiner), in which the Office states:

> The reference required by 37 CFR 1.78(a)(2) or (a)(5) must be included in an application data sheet (37 CFR 1/76), or the specification must contain, or be amended to contain, such reference in the first sentence following the title. Previously the Office indicated that if an applicant includes a benefit claim in the application but not in the manner specified by 37 CFR 1.78 (a) (e.g., if the claim is included in an oath or declaration or the application transmittal letter) within the time period set forth in 37 CFR 1.78(a), the Office will not require a petition under 37 CFR 1.78(a) and the surcharge under 37 CFR 1.17(t) to correct the claim if the information concerning the claim was recognized by the Office as shown by its inclusion on the filing receipt. . . . The Office will continue to follow this practice. (emphasis added).

Hence, Applicants respectfully request that the Office accept Applicants' priority claim without requiring either the petition as set forth in 37 CFR 1.78(a)(2) or the fee as set forth in 37 CFR 1.17(t) and amend the specification as requested in this response and request for reconsideration.

Amendments to the Claims:

Claims 25-26, and 35-36 are cancelled herein without disclaimer or prejudice to pursuing the inventions of claims 25-26 and 35-36 in a continuing application. Claim 33

is amended herein such that it no longer depends from cancelled claim 22 but now properly depends from claim 27. No new matter is added by this amendment.

Claim Rejections under 35 U.S.C. § 101

Utility:

The Office has maintained rejection of claims 25-34 under 35 U.S.C. § 101 alleging that the claimed invention is not supported by either a substantial asserted utility or a well established utility.

In particular, the Office maintains that it has not set the standard for satisfying the utility requirement too high because according to the Office, "the only nucleic acid that is overexpressed in tumor is SEQ ID NO:68 and there is no indication that SEQ ID NO: 69 or any other polypeptide is overexpressed in tumors." The Office maintains that absent explicit evidence that the claimed polypeptide is overexpressed, the utility of a PRO357 polypeptide as a diagnostic marker is unpredictable.

Applicants respectfully disagree. As previously argued, at pages 119 and 137 of the specification, Applicants assert a diagnostic utility for the claimed polypeptide. This assertion creates a presumption of utility sufficient to satisfy the utility requirement of 35 U.S.C. § 101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope." *In re Langer*, 183 USPQ 288, 297 (CCPA 1974). *See also In re Jolles*, 206 USPQ 885 (CCPA 1980); *In re Irons*, 144 USPQ 351 (9165); *In re Sichert*, 196 USPQ 209, 212-213 (CCPA 1977). The evidentiary standard to be used when determining whether one skilled in the art would question the objective truth of Applicants' statement of utility or its scope is a preponderance of the totality of the evidence under consideration. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). Thus, to overcome the presumption of truth that an assertion of utility by the Applicant enjoys, the Office must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. For the reasons explained below, Applicants respectfully maintain that the Office has not met this burden.

Previously, Applicants relied on Pollack, Orntoft, Hyman, Bermont, Varis, and Hu as support for the diagnostic utility of the claimed invention. Applicants argued that consideration of the totality of this evidence clearly demonstrated the claimed invention is supported by a specific, substantial, and credible utility and that the maintained rejection of the present claims for lack of utility is improper and should be withdrawn.

The Office disagrees that this evidence cited by Applicants demonstrates that the claimed polypeptide is supported by a specific, substantial, and credible utility. In particular, the Office maintains that one of ordinary skill in the art would question the objective truth of Applicants' assertion of utility because, according to the Office, the evidence cited by Applicants, as well as the Pennica reference cited by the Examiner, shows unpredictability of protein expression correlated to mRNA levels. For example, the Office rejects Applicants' reliance on the Hu reference because, according to the Office, Hu only demonstrates a correlation between gene amplification and protein overexpression for "one protein overexpressed of 18 identified." Similarly, the Office rejects Applicants' reliance on Bermont, arguing that "Bermont . . . only show(s) p185 as the overexpressed protein." Additionally, the Office does not find Orntoft to be persuasive evidence in support of Applicants' assertion of utility. In particular, the Office alleges that Orntoft teaches that protein degradation, which is related to protein half-life, may be important in determining whether protein expression levels correlate to gene amplification levels. The Office notes that the half life of PRO357 is unknown and therefore, concludes that based on Orntoft, one of ordinary skill in the art would doubt Applicants' assertion of utility. Finally, the Office rejects Applicants' reliance on Pollack, Hyman, and Varis arguing that these references only teach correlation of gene amplification levels with mRNA levels. Thus, the Office rejects the evidence submitted by Applicants and argues that this evidence, as well as the Pennica reference cited by the Examiner, demonstrates the unpredictability in the art. With regard to utility based on a correlation between gene amplification and protein overexpression, the Office asserts that the correlation needs to be determined on a case by case basis.

Applicants respectfully disagree. In support of Applicants assertion of utility, it is first noted that statistical certainty is not required to satisfy 35 U.S.C. § 101. *Nelson v.*

Bowler, 626 F.2d 853, 856-857, 205 USPQ 881, 883-884 (CCPA 1980). Thus, Applicants maintain that even though every reference may not teach 100% correlation between gene amplification levels and protein overexpression in cancerous tissues, in view of the references cited by Applicants, one of ordinary skill in the art would not doubt the truth of Applicants' assertion of utility.

Specifically, although the Hu reference Applicants previously submitted for the Office's consideration (Hu et al., "Profiling of Differentially Expressed Cancer-related Genes in Espohageal Squamous Cell Carcinoma (ESCC) Using Human Cancer cDNA Arrays: Overexpression of Oncogene *Met* correlates with tumor differentiation in ESCC." 2001. *Clin. Cancer Research,* 7:3519-3525), mentions that 18 genes were identified as being differentially expressed in cancerous esophageal tissue compared to non-cancerous esophageal tissue, the protein expression levels were only evaluated for *one* of the genes, *Met.* Significantly, Hu teaches a positive correlation between gene amplification and protein overexpression of this gene in cancerous tissues. Additionally, Hu references a related article wherein Hu reported that they examined the protein expression levels for four additional genes that previously were found to be amplified in esophageal cancer. *See* Hu at page 3523, referring to results reported at Hu *et al.,* "Identification of Differentially Expressed Genes in Espohageal Squamous Cell Carcinoma (ESCC) by cDNA Expression Array: Overexpression of *Fra-1, Neogenin, Id-1,* and *CDC25B* Genes in ESCC." 2001. *Clin. Cancer Research,* 7:2213-2221 (attached hereto as Appendix 3 for the Examiner's convenience).

The second Hu reference reports that they first used a cDNA expression array hybridization assay to identify the 18 genes that were discovered to be differentially expressed in ESCC compared with normal esophageal epithelium. In the second Hu reference, four of these eighteen genes, *Fra-1, Neogenin, ID-1,* and *CDC25B* were selected for more detailed study of their protein expression levels. Specifically, the second Hu reference reports that "the protein products of *Fra-1, Neogenin, Id-1,* and *CDC25B* were found to be overexpressed in both the ESCC cell lines and their corresponding primary tumors . . . validating the cDNA array results." Hu at 2216.

Thus, although Hu *et al.* identified 18 genes that were differentially expressed (*e.g.* amplified) in cancerous esophageal tissue compared to normal esophageal tissue, Hu *et al.* only examined the protein expression patterns of five of these 18 (*Met, Fra-1, Neogenin, Id-1,* and *CDC25B*). Significantly, 100% (5/5) of the genes examined demonstrated a correlation between differential gene expression and protein expression levels. Thus, Applicants respectfully submit that in view of the teachings of Hu, one of ordinary skill in the art would not doubt Applicants' assertion of utility. Rather, the teachings of Hu *et al.* clearly support Applicants' assertion of utility. Applicants maintain that the Office has not met its burden of establishing that it is more likely than not that, in view of the teachings of Hu, one of ordinary skill in the art would question Applicants' assertion of utility.

Further, as the Office acknowledges, Orntoft teaches correlation of gene amplification and protein overexpression in human bladder cancer. Indeed, Orntoft reports that for human bladder tumors, gene amplification in the bladder tumor tissues showed a "*striking correspondence*" to protein expression levels. Orntoft at 44 (emphasis added). In contrast, the reference Orntoft cites for the proposition that protein degradation may be important for proteins with a short half-life only looks at whether there is a correlation between gene and protein expression levels in *non-cancerous* liver cells or non-human yeast cells. *See* Anderson, L and Seilhamer, J. 1997. "A comparison of selected mRNA and protein abundances in human liver." *Electrophoresis,* 18(3-4):533-7; Ideker *et al.* 2001. "Integrated genomic and proteomic analyses of a systematically perturbed metabolic network." *Science,* 292:929-934.

The claims of the present invention are directed to a polypeptide encoded by a nucleic acid sequence that is amplified in lung and colon *tumors*. *See e.g.* Example 28: Gene Amplification, pages 119-137 of the specification. Thus, Applicants respectfully submit that the teachings of Orntoft are more applicable to the present invention than the teachings of the Anderson and Ideka references mentioned in Orntoft. Hence, although one of ordinary skill in the art may appreciate the significance of protein degradation and post-translational modification as mentioned by Orntoft, Applicants respectfully maintain that based on the *entirety* of the teachings of Orntoft, one of ordinary skill in

the art would not doubt Applicants' assertion of a utility for the claimed polypeptide. Significantly, according to the MPEP, where an Applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed as "wrong" even where there may be some reason to question the assertion. MPEP § 2107.02.

Applicants rely on Bermont as additional evidence that the claimed polypeptide is supported by a specific, substantial, and credible utility because gene amplification of nucleic acids in cancerous tissues (*e.g.* c-erbB-2 in breast cancer tissues or PRO357 in lung and colon cancer tissues) correlates with protein overexpression in those tissues. Indeed, Bermont reports that 100% of the overexpressed p185 protein in 106 breast cancer samples studied also displayed c-erbB-2 amplification. *See* Bermont et al., "Relevance of p185 HER-2/neu oncoprotein quantification in human primary breast carcinoma." *Breast Cancer Res Treat.* 2000 63(2):163-9. Thus, Applicants respectfully submit that the Office has not satisfied its burden of demonstrating that one of ordinary skill in the art, in view of the Bermont reference, would more likely than not doubt Applicants' assertion of utility.

Further, even assuming *arguendo* that Pollack, Hyman, and Varis only teach correlation between gene amplification and mRNA levels, these references still do not establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of Applicants' assertion of utility. Nor does the Pennica *et al.* reference. In particular, for 2 of 3 genes studied, Pennica reported positive correlation between gene amplification and protein overexpression. Pennica also reported that the data showing that the expression levels of the third gene did not positively correlate with protein overexpression levels might be inaccurate. *See* Pennica at 14722. Thus, because the RNA expression pattern of this gene cannot be accurately correlated with the protein expression pattern of this gene, this result should be disregarded. Therefore, particularly in light of the references discussed above, the Office's reliance on the Pennica reference does not establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of Applicants' assertion of utility.

Significantly, a 35 U.S.C. § 101 rejection should only be sustained where the asserted utility violates a scientific principle or is *wholly* inconsistent with contemporary knowledge in the art. *In re Gazave,* 379 F.2d 973, 978 (CCPA 1967). As explained above, Applicants' asserted utility does not violate any scientific principles, nor is it inconsistent with contemporary knowledge. Rather, Applicants' asserted utility is consistent with the evidence presented in the references cited by Applicants (*e.g.* Hu reports 100% correlation between differential gene expression levels and protein expression levels for 5 genes in cancerous esophageal tissue; Orntoft reports a striking correlation for gene amplification and protein overexpression in bladder cancer samples; and Bermont teaches a 100% correlation between gene amplification of c-erbB-2 and p185 overexpression in breast cancer tumors). Moreover, the Office has not satisfied its burden of establishing that it is more likely than not that one of ordinary skill in the art would doubt the truth of Applicants' assertion of utility for the claimed polypeptide. Thus, Applicants maintain that this rejection is improper and should be withdrawn.

Furthermore, "[a]n applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement." MPEP § 2107. In addition to asserting the diagnostic utility of the claimed polypeptide discussed above, Applicants assert another utility of PRO357. In particular, at page 80, lines 25-26 of the specification, Applicants assert that PRO357 polypeptides can be used in competitive binding assays with the acid labile subunit of insulin-like growth factor (ALS).

Use of the PRO357 polypeptide in competitive binding assays with ALS is a specific utility. "Specific utility" is defined as:

> [a] utility that is *specific* to the subject matter claimed. This contrasts with a *general* utility that would be applicable to the broad class of the invention. For example, a polynucleotide whose use is disclosed simply as a 'gene probe' or 'chromosome marker' would not be considered to be *specific* in the absence of a disclosure of a specific DNA target. Similarly, a general statement of diagnostic utility, such as diagnosing an unspecified disease, would ordinarily be insufficient absent a disclosure of what condition can be diagnosed.

Revised Interim Utility Guidelines Training Materials, pgs. 5-6 (http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf). Utility of the PRO357 polypeptide in competitive binding assays with ALS is specific because it is based on the amino acid sequence and structure of the PRO357 polypeptide, as well as on the homology PRO357 shares with ALS. *See* page 58, lines 3-5 of the specification.

A competitive binding assay is an immunoassay reaction which is based upon the competition of labeled and unlabeled ligand for a limited number of binding sites on the binder. A fixed amount of the labeled ligand (PRO357) and a variable amount of the unlabeled ligand (ALS) are incubated with the binder. Following the law of mass action, the amount of labeled ligand which can bind to the binder is a function of the total concentration of labeled and unlabeled ligand. As the concentration of unlabeled ligand is increased, less labeled ligand can bind to the binder and the measured response is decreased. Thus, a competitive binding assay using PRO357 and ALS can provide information regarding the concentration of ALS in a sample. This information is very useful because ALS levels in acromegalic patients are about twice those present in normal subjects. *See* Arosio *et al.,* 2001. "Diagnostic Value of the Acid-Labile Subunit in Acromegaly: Evaluation in Comparison with Insulin-Like Growth Factor (IGF) I, and IGF-Binding Protein-1, -2, and -3." *J. Clin. Endocrinology,* 86(3):1091-1098. (Attached hereto as Appendix 4). Hence, the utility of PRO357 in a competitive binding assay with ALS is specific because the results of this assay can be used to diagnose acromegaly.

Use of the PRO357 polypeptide in competitive binding assays with ALS also is a substantial utility. "Substantial utility" is defined as:

> a utility that defines a 'real world' use. An assay that measures the presence of a material which has a stated correlation to a predisposition to the onset of a particular disease condition would also define a "real world" context of use in identifying potential candidates for preventive measure or further monitoring.

Revised Interim Utility Guidelines Training Materials, pg. 6 (http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf). Utility of the PRO357 polypeptide in competitive binding assays with ALS is substantial because, as explained above, the competitive

binding assay measures the presence of ALS, which has a stated correlation to acromegaly.

Further, use of PRO357 polypeptides in competitive binding assays with ALS is a credible utility:

> Where an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office personnel as being 'wrong'. Rather, Office personnel must determine if the assertion of utility is credible (*i.e.,* whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided). An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. Credibility as used in this context refers to the reliability of the statement based on the logic and facts that are offered by the applicant to support the assertion of utility. A *credible* utility is assessed from the standpoint of whether a person of ordinary skill in the art would accept that the recited or disclosed invention is currently available for such use. For example, no perpetual motion machines would be considered to be currently available. However, nucleic acids could be used as probes, chromosome markers, or forensic or diagnostic markers. Therefore the credibility of such an assertion would not be questioned, although such a use might fail the *specific* and *substantial* tests.

Revised Interim Utility Guidelines Training Materials, pg. 5 (http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf). Utility of the PRO357 polypeptide in competitive binding assays with ALS is credible because neither the logic underlying this assertion is flawed, nor are the facts inconsistent with this logic. At page 58, lines 3-5 of the specification, Applicants disclose that BLAST and FastA sequence alignment computer programs illustrate that various portions of the PRO357 polypeptide have homology with ALS. For example, both PRO357 polypeptide and ALS have leucine rich repeat sequences of 24 amino acids and both have several asparagine-linked glycosylation sites (N-glycosylation sites). It is highly significant that both proteins share N-glycosylation sites, as these sites are important for binding of ALS to its binding partner, IGFBP-3. *See* Suwanichkul *et al.,* 2000. "Conservation of a Growth Hormone-Responsive Promoter Element in the Human and Mouse Acid-Labile Subunit Genes." *Endocrinology,* 141 (2):833-838 (attached hereto as Appendix 5). Indeed, these N-glycosylation sites are a *requirement* for ALS binding to IGFBP-3 and IGF. *See* Janosi

et al., 1999. "*N*-linked Glycosylation and Sialylation of the Acid-Labile Subunit." *The J. of Biological Chemistry,* 274(9):5292-5298 (attached hereto as Appendix 6). Thus, based on the homology between PRO357 polypeptides and ALS, and most significantly, based on the presence of N-glycosylation sites on PRO357, which are required for ALS binding, it is logical that PRO357 may be used in competitive binding assays with ALS. Therefore, this utility of PRO357 polypeptides also is credible.

Finally, use of PRO357 polypeptides in competitive binding assays with ALS is a well-established utility. A "well established" utility is a:

> specific, substantial, and credible utility which is well known, immediately apparent, or implied by the specification's disclosure of the properties of the material, alone or taken with the knowledge of one skilled in the art.

Revised Interim Utility Guidelines Training Materials, pg. 7 (http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf). Utility of PRO357 polypeptides in competitive binding assays with ALS, is a well-established utility because, as explained above, this utility is specific, substantial, and credible and the specification discloses this utility at page 80, lines 25-26.

Thus, even if the Office rejects the arguments made herein supporting the diagnostic utility of the claimed PRO357 polypeptide, a rejection of presently pending claims 27-34 under 35 U.S.C. § 101 for lack of utility still is improper because these claims are supported by an additional utility of PRO357. Specifically, as explained above, PRO357 polypeptides may be used in competitive binding assays with ALS. Hence, Applicants respectfully request that this ground of rejection be withdrawn.

35 U.S.C. § 112 ¶ 1, Enablement-Utility

The Examiner has rejected claims 25-36 under 35 U.S.C. § 112 ¶1, alleging that because the claimed invention is not supported by either a specific asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention. As discussed in the remarks above, addressing the rejection under 35 U.S.C. § 101 for lack of utility, Applicants respectfully submit that the claimed polypeptide is supported by a specific, substantial, and credible utility. Thus, Applicants respectfully

request the Examiner reconsider and withdraw the rejection of claims 25-34 under 35 U.S.C. § 112 ¶1 for their alleged inadequate disclosure on how to use the claimed invention.

Claim rejections under 35 U.S.C. § 112, first paragraph

Written Description:

The Office maintains rejection of Claims 25-26 and 33-34 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the invention.

In order to expedite prosecution of this application, Applicants have herein cancelled claims 25-26 without disclaimer or prejudice to pursuing the invention of those claims in a continuing application. Additionally, Applicants have herein amended the dependency of claims 33-34 such that they no longer depend from rejected claims 25-26, but now depend from claim 27, which is not rejected for lack of a written description. Hence, Applicants respectfully submit they have overcome this ground of rejection and respectfully request it be withdrawn.

Enablement:

The Office also maintains rejection of Claims 25-36 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention.

In order to expedite prosecution of this application, Applicants have herein cancelled claims 25-26 without disclaimer or prejudice to pursuing the invention of those claims in a continuing application. Hence, Applicants respectfully submit they have overcome this ground of rejection and respectfully request it be withdrawn.

Conclusion

Applicants believe that currently pending Claims 27-34 are patentable. Applicants respectfully request the Examiner grant allowance of this application. The Examiner is invited to contact the undersigned attorney for Applicants via telephone if such communication would expedite the prosecution this application.

Respectfully submitted,

C. Noel Kaman

C. Noel Kaman
Registration No. 51,857
Attorney for Applicant

BRINKS HOFER GILSON & LIONE
P.O. BOX 10395
CHICAGO, ILLINOIS 60610
(312) 321-4200

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
Washington, DC 20231
www.uspto.gov

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
09/943,780	08/30/2001	1642	710	P2548P1C10	34	13	2

CONFIRMATION NO. 2570

CORRECTED FILING RECEIPT

OC000000009593127

28442
BRINKS HOFER GILSON & LIONE
P.O. BOX 10395
CHICAGO, IL 60610

Date Mailed: 03/05/2003

Receipt is acknowledged of this regular Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections, facsimile number 703-746-9195. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

Kevin P. Baker, Darnestown, MD;
David Botstein, Belmont, CA;
Dan L. Eaton, San Rafael, CA;
Napoleone Ferrara, San Francisco, CA;
Ellen Filvaroff, San Francisco, CA;
Mary E. Gerritsen, San Mateo, CA;
Audrey Goddard, San Francisco, CA;
Paul J. Godowski, Burlingame, CA;
J. Christopher Grimaldi, San Francisco, CA;
Austin L. Gurney, Belmont, CA;
Kenneth J. Hillan, San Francisco, CA;
Ivar J. Kijavin, Pacifica, CA;
Mary A. Napier, Hillsborough, CA;
Margaret Ann Roy, San Francisco, CA;
Daniel Tumas, Orinda, CA;
William I. Wood, Hillsborough, CA;

Assignment For Published Patent Application

Genentech, Inc.;

Domestic Priority data as claimed by applicant

This application is a CON of 09/866,028 05/25/2001
which claims benefit of 60/067,411 12/03/1997
and claims benefit of 60/069,334 12/11/1997
and claims benefit of 60/069,335 12/11/1997
and claims benefit of 60/069,278 12/11/1997
and claims benefit of 60/069,425 12/12/1997

and claims benefit of 60/069,696 12/16/1997
and claims benefit of 60/069,694 12/16/1997
and claims benefit of 60/069,702 12/16/1997
and claims benefit of 60/069,870 12/17/1997
and claims benefit of 60/069,873 12/17/1997
and claims benefit of 60/068,017 12/18/1997
and claims benefit of 60/070,440 01/05/1998
and claims benefit of 60/074,086 02/09/1998
and claims benefit of 60/074,092 02/09/1998
and claims benefit of 60/075,945 02/25/1998
and claims benefit of 60/112,850 12/16/1998
and claims benefit of 60/113,296 12/22/1998
and claims benefit of 60/146,222 07/28/1999

Foreign Applications

UNITED STATES OF AMERICA PCT/US98/19330 09/16/1998
UNITED STATES OF AMERICA PCT/US98/25108 12/01/1998
UNITED STATES OF AMERICA PCT/US99/12252 06/22/1999
UNITED STATES OF AMERICA PCT/US99/21090 09/15/1999
UNITED STATES OF AMERICA PCT/US99/28409 11/30/1999
UNITED STATES OF AMERICA PCT/US99/28313 11/30/1999
UNITED STATES OF AMERICA PCT/US99/28301 12/01/1999
UNITED STATES OF AMERICA PCT/US99/30095 12/16/1999
UNITED STATES OF AMERICA PCT/US00/03565 02/11/2000
UNITED STATES OF AMERICA PCT/US00/04414 02/22/2000
UNITED STATES OF AMERICA PCT/US00/05841 03/02/2000
UNITED STATES OF AMERICA PCT/US00/08439 03/30/2000
UNITED STATES OF AMERICA PCT/US00/14042 05/22/2000
UNITED STATES OF AMERICA PCT/US00/20710 07/28/2000
UNITED STATES OF AMERICA PCT/US00/32678 12/01/2000
UNITED STATES OF AMERICA PCT/US01/06520 02/28/2001

If Required, Foreign Filing License Granted: 11/13/2001

Projected Publication Date: 02/21/2002

Non-Publication Request: No

Early Publication Request: No

Title

Secreted and transmembrane polypeptides and nucleic acids encoding the same

Preliminary Class

514

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Office of Export Administration, Department of Commerce (15 CFR 370.10 (j)); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

United States Patent and Trademark Office OG Notices: 18 March 2003

Claiming the Benefit of a Prior-Filed Application under 35 U.S.C. 119(e), 120, 121, and 365(c)

Summary: This notice clarifies how benefit claims under 35 U.S.C. 119(e), 120, 121 and 365(c) must be presented in applications in order to be in compliance with the relevant statute and patent regulations, and accepted by the United States Patent and Trademark Office (Office).

35 U.S.C. 120 Benefit Claims

Benefit claims under 35 U.S.C. 120 must include a specific reference to the earlier filed (nonprovisional) application for which a benefit is sought. A "specific reference" requires: (1) the identification of the prior (nonprovisional) application by application number; and (2) an indication of the relationship between the nonprovisional applications, except for the benefit claim to the prior application in a continued prosecution application (CPA). The relationship between any two nonprovisional applications will be an indication that the later-filed nonprovisional application is either a continuation, divisional, or continuation-in-part of the prior-filed nonprovisional application. When there are benefit claims to multiple prior nonprovisional applications (e.g., a string of prior nonprovisional applications), the relationship must include an identification of each nonprovisional application as either a continuation, divisional, or continuation-in-part application of a specific prior nonprovisional application for which a benefit is claimed. The identification is needed in order to be able to verify if copendency exists throughout the entire chain of prior nonprovisional applications.

35 U.S.C. 119(e) Benefit Claims

Benefit claims under 35 U.S.C. 119(e) must include a specific reference to the earlier filed provisional application for which a benefit is sought. A specific reference in this situation, however, only requires identification of the prior provisional application by the application number. No relationship between the subject nonprovisional application and the prior provisional application should be specified. If the subject nonprovisional application was not filed within twelve months of the filing date of the provisional application, the subject application must also include a benefit claim under 35 U.S.C. 120 to an intermediate prior nonprovisional application that was filed within twelve months of the filing date of the provisional application. Therefore, in addition to the identification of the provisional application, the proper benefit claim for this situation must also identify the intermediate prior nonprovisional application that is directly claiming the benefit of the provisional application, and indicate the relationship between the nonprovisional applications (e.g., an indication that the subject application is a continuation of the intermediate prior nonprovisional application).

Statement of the Problem

Background: Some applicants have been submitting patent applications which include, or are amended to include, at the beginning of the specification, a statement that benefits are claimed under 35 U.S.C. 119(e) and/or 120 to prior applications, followed by a listing of many prior nonprovisional and provisional applications. The listings do not indicate: (1) the specific relationship (i.e., continuation, divisional, or continuation-in-part) between the nonprovisional applications, as required by 37 CFR 1.78(a)(2)(i); and/or (2) each nonprovisional application which is directly claiming priority to a provisional application. Without such information, the Office does not have sufficient information to enter the benefit claims into the Office's computer database.

When entering benefit claims for an application into the Office's database, the relationship (i.e., continuation, divisional or continuation-in-part) between the nonprovisional applications is required. Further, the system will not accept any benefit claim to a provisional application if the provisional application was filed more than twelve months prior to the filing date of the subject application, unless the applicant clearly identifies, and claims the benefit of, a prior nonprovisional application that was filed within twelve months of the filing date of the provisional application. Accordingly, if benefit claims are presented without all the required information, the Office will not be able to enter such benefit claims into the Office's database, the filing receipt will not reflect the prior application(s), and the projected publication date will not be calculated as a function of an earlier application's filing date.

The specific relationships between (each of) the nonprovisional applications in a chain of nonprovisional applications are also important because such information will be printed in the application publication, and/or patent. Furthermore, the designation of an application as a continuation (rather than as a continuation-in-part) is an indication that the entire invention claimed in an application has support in the prior application, whereas the designation of an application as a continuation-in-part is an indication that the claimed invention is not entirely supported by the prior application. Thus, the specific relationship between nonprovisional applications in a chain of benefit claims, and the indication of the specific nonprovisional application(s) that is directly claiming the benefit of a provisional application, will provide the information that is needed by examiners and the public in order to determine the effective prior art date of the application publication, or patent, under 35 U.S.C. 102(e).

When benefit claims are required to, but do not, include: (1) an identification of (all) intermediate benefit claims, and/or (2) the relationship between nonprovisional applications, the Office may not be able to publish applications promptly after the expiration of a period of eighteen months from the earliest filing date for which a benefit is sought under title 35, United States Code (eighteen-month publication), nor have the accuracy desired of such benefit claims in application publications. Further, the objection (by the Office), correction (by applicant), and review/entry of changes (by the Office) cycle for non-compliant benefit claims is a burdensome effort on both applicants and the Office that can be totally avoided if such benefit claims are properly submitted the first time. Accordingly, it is hoped that applicants will submit benefit claims with all the required information as set forth in this notice and, correspondingly, avoid submitting non-compliant benefit claims that leads to extra work for both the Office and applicants.

Procedures for Making Proper Benefit Claims

Part I: Reference to Prior Nonprovisional Application(s) Per 37 CFR 1.78(a)(2)(i) Must Include Identification of, and Relationship Between, Applications

35 U.S.C. 120 provides that no application shall be entitled to the benefit of an earlier filed application unless it contains, or is amended to contain, a specific reference to the earlier filed application. The specific reference required by 35 U.S.C. 120 is the reference required by 37 CFR 1.78(a)(2). 37 CFR 1.78(a)(2)(i) requires that any nonprovisional application that claims the benefit of one or more prior-filed copending nonprovisional applications, or international applications designating the United States, must contain, or be amended to contain, a reference to each such prior-filed application, identifying it by application number (consisting of the series code and serial number), or international application number and international filing date, and indicating the relationship of the applications. 37 CFR 1.78(a)(2)(iv) also provides that a request for a continued prosecution application (CPA) under 37 CFR 1.53(d) is the specific reference required under 35 U.S.C. 120 to the prior-filed application. Therefore, except for the benefit claim to the prior-filed application in a CPA, benefit claims under 35 U.S.C. 120, including claims under 35 U.S.C. 121 and 365(c), must not only identify the earlier application by application number, or by international application number and international filing date, but they must also indicate the relationship between the applications.

Examples

The relationship between the applications is whether the subject application is a continuation, divisional, or continuation-in-part of a prior-filed nonprovisional application. An example of a proper benefit claim is: "This application is a continuation of Application No. 10/ - , filed - ." A benefit claim that merely states: "This application claims the benefit of Application No. 10/ - , filed - ." does not comply with 37 CFR 1.78(a)(2)(i), since the relationship between the applications is not stated. In addition, a benefit claim that merely states: "This application is a continuing application of Application No. 10/ - , filed - ." does not comply with 37 CFR 1.78(a)(2)(i) since the proper relationship, which includes the type of continuing application, is not stated. It is also noted that the status of each nonprovisional parent application (if it is patented or abandoned) should also be indicated, following the filing date of the parent nonprovisional application. An example of a proper benefit claim of a prior national stage of an international application is "This application is a continuation of U.S. Application No. X, which is the National Stage of International Application No. PCT/US - / - , filed - ." For additional examples of proper benefit claims, see Manual of Patent Examining Procedure (8th ed., August 2001)(MPEP), Section 201.11, Reference to First Application. Section 201.11 of the MPEP will be revised in the upcoming revision to reflect the clarification made in this notice about the required manner of making proper claims for the benefit of prior nonprovisional and provisional applications.

As stated previously, to specify the relationship between the nonprovisional applications, applicant must specify whether the subject application is a continuation, divisional, or continuation-in-part of the prior nonprovisional application. Note that the terms are exclusive. An application cannot be, for example, both a continuation

and a divisional, or a continuation and a continuation-in-part, of the same parent application. Moreover, if the benefit of more than one nonprovisional parent application is claimed, the relationship must include an identification of each nonprovisional application as a continuation, divisional, or continuation-in-part application of the immediate prior nonprovisional application for which a benefit is claimed in order to establish co-pendency throughout the entire chain of prior-filed parent nonprovisional applications. For example, the following two statements are improper: "This application claims the benefit of Application Nos. C, B, and A." and "This application is a continuing application of Application Nos. C, B, and A." On the other hand, the following statement is proper and acceptable: "This application is a continuation of Application No. C, filed - , which is a continuation of Application No. B, filed - , which is a continuation of Application No. A, filed - ."

Sanctions for Making Improper Benefit Claims to Nonprovisional Applications

Any benefit claim under 35 U.S.C. 120, 121 or 365(c) that does not identify a prior application and also specify a relationship between each of the applications will not be in compliance with 37 CFR 1.78(a)(2)(i), and will not be considered to contain a specific reference to a prior application as required by 35 U.S.C. 120. Such a benefit claim will not be recognized by the Office and will not be included on the filing receipt for the application, even if the claim appears in the first sentence of the specification or an application data sheet (37 CFR 1.76), because the Office does not have sufficient information to enter the benefit claim into the Office's database. As a result, publication of the application will not be scheduled as a function of the prior application's filing date. The Office plans to notify applicants on, or with, the filing receipt that a benefit claim may not have been recognized because it did not include the proper reference. Applicants are advised that only the benefit claims that are listed on the filing receipt have been recognized by the Office. Since the filing receipt and the notification will usually be provided to the applicant shortly after the filing of the application, applicants should have sufficient opportunity to submit the proper benefit claims within the time period set in 37 CFR 1.78(a)(2)(ii) and thus avoid the need to submit a petition under 37 CFR 1.78(a)(3) and the surcharge set forth in 37 CFR 1.17(t). Failure to timely submit the reference required by 37 CFR 1.78(a)(2)(i) is considered a waiver of any benefit claim under 35 U.S.C. 120, 121 or 365(c) unless a petition to accept an unintentionally delayed claim under 37 CFR 1.78(a)(3), the surcharge set forth in 37 CFR 1.17(t), and the required reference, including the relationship of the applications (unless previously submitted) are filed. For example, if a benefit claim is submitted without the specific relationship between the nonprovisional applications before the expiration of the period, and the specific relationship between the nonprovisional applications is subsequently submitted after the expiration of the period, a petition and the surcharge would be required.

Part II: Reference to Prior Provisional Application(s) Per 37 CFR 1.78(a)(5)(i) Should Only Include Identification of Prior Provisional Application(s)

When the domestic benefit of a prior provisional application is being claimed under 35 U.S.C. 119(e), however, the relationship between the two applications should not be specified. 35

U.S.C. 119(e) provides that a nonprovisional application claiming the benefit of a provisional application must be filed within twelve months of the provisional application and must contain, or be amended to contain, a specific reference to the provisional application. The specific reference required by 35 U.S.C. 119(e) is the reference required by 37 CFR 1.78(a)(5). 37 CFR 1.78(a)(5)(i) requires that any nonprovisional application, or international application designating the United States, claiming the benefit of one or more prior-filed provisional applications must contain, or be amended to contain, a reference to each such prior-filed provisional application identifying it by provisional application number. No relationship should be specified whenever a claim for the benefit of a provisional application under 35 U.S.C. 119(e) is made.

If a relationship between a nonprovisional application and a prior provisional application is submitted, however, it may be unclear whether applicant wishes to claim the domestic benefit of the provisional application under 35 U.S.C. 119(e), or the benefit of an earlier application's filing date under 35 U.S.C. 120. Thus, applicants seeking to claim the domestic benefit of a provisional application under 35 U.S.C. 119(e) should not state that the application is a "continuation" of a provisional application, nor should it be stated that the application claims benefit under 35 U.S.C. 120 of a provisional application. If such a claim is submitted in an application transmitted to the Office other than through the Electronic Filing System, it will be entered into the Office computer system as a claim to the "benefit" of the provisional application. Although 35 U.S.C. 120 does not preclude a benefit claim to a provisional application (that is, one could obtain the benefit under 35 U.S.C. 120 of a prior filed provisional application), such a benefit claim under 35 U.S.C. 120 is not recommended as such a claim may have the effect of reducing the patent term, as the term of a patent issuing from such an application may be measured from the filing date of the provisional application pursuant to 35 U.S.C. 154(a)(2). Instead, applicants should state "This application claims the benefit of U.S. Provisional Application No. 60/ - , filed - ", or "This application claims the benefit of U.S. Provisional Application No. 60/ - , filed - , and U.S. Provisional Application 60/ - , filed - ." See MPEP 201.11, Reference to First Application (8th ed., August 2001).

Part III: If Benefit is Claimed of a Prior Provisional Application Which was Filed More Than One Year Before the Subject Application, Then Each Prior Nonprovisional Application(s) Claiming Benefit of the Provisional Must be Specified

Any nonprovisional application that directly claims the benefit of a provisional application under 35 U.S.C. 119(e) must be filed within twelve months from the filing date of the provisional application. As noted above, an application that itself directly claims the benefit of a provisional application should identify, but not specify the relationship to, the provisional application. If the subject nonprovisional application is not filed within the twelve month period, however, it still may claim the benefit of the provisional application via an intermediate nonprovisional application under 35 U.S.C. 120. The intermediate nonprovisional application must have been filed within twelve months from the filing date of the provisional application and the intermediate nonprovisional application must have claimed the benefit of the provisional application. Further, it must be clearly indicated that the intermediate nonprovisional application is

claiming the benefit under 35 U.S.C. 119(e) of the provisional application. This identification of the intermediate nonprovisional application is necessary so that the Office can determine whether the intermediate nonprovisional application was filed within twelve months of the filing date of the provisional application, and thus, whether the benefit claim is proper.

Examples

Applicant should state such a benefit claim as follows: "This application is a continuation of Application No. C, filed - , which is a continuation of Application No. B, filed - , which claims the benefit of U.S. Provisional Application No. A, filed - ." A benefit claim that merely states "This application claims the benefit of nonprovisional Application No. C, filed - , nonprovisional Application No. B, filed - , and provisional application No. A, filed - " would be improper where the subject application was not filed within twelve months of the provisional application.

Where the benefit of more than one provisional application is being claimed, the intermediate nonprovisional application(s) claiming the benefit of each provisional application must be clearly indicated. Applicant should state, for example, "This application is continuation of Application No. D, filed - , which is a continuation-in-part of Application No. C, filed - , Application No. D claims the benefit of U.S. Provisional Application No. B, filed - , and Application No. C claims the benefit of U.S. Provisional Application No. A, filed - ." An example of a proper benefit claim of a prior national stage of an international application, which claims the priority to a provisional application, is "This application is a continuation of U.S. Application No. Y, which is the National Stage of International Application No. PCT/US - / - , filed - , which claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Application X, filed - ."

Sanctions for Making Improper Benefit Claims to Provisional Applications

If a benefit claim to a provisional application is submitted without an indication that an intermediate nonprovisional application directly claims the benefit of the provisional application and the instant nonprovisional application is not filed within the twelve month period, or the relationship between nonprovisional applications is not indicated, the Office will not have sufficient information to enter the benefit claim into the computer database. Therefore, the Office will not recognize such a benefit claim, and will not include the benefit claim on the filing receipt. The Office plans to notify applicants on, or with, the filing receipt that a benefit claim may not have been recognized because information regarding the intermediate nonprovisional application(s) and/or the relationship between each nonprovisional application have not been provided. Applicants are advised that only the benefit claims that are listed on the filing receipt have been recognized by the Office. Since the filing receipt and the notification will usually be provided to the applicant shortly after the filing of the application, applicants should have sufficient opportunity to submit the proper benefit claims within the time period set in 37 CFR 1.78(a) and thus avoid the need to submit a petition under 37 CFR 1.78(a) and the surcharge set forth in 37 CFR 1.17(t). Failure to timely submit the reference required by 37 CFR 1.78(a) is considered a waiver of any benefit claim under 35 U.S.C. 119(e),

120, 121 or 365(c) unless a petition under 37 CFR 1.78(a), the surcharge set forth in 37 CFR 1.17(t), identification of the intermediate nonprovisional application which claims the benefit to the provisional application, and the relationship between each nonprovisional application are filed.

Part IV: Office Practice to Not Require Petition and Surcharge if Benefit Claim is Not Present in the Proper Place But is Recognized By Office Continues But Applicants Are Advised That Proper Reference Must be Presented

The reference required by 37 CFR 1.78(a)(2) or (a)(5) must be included in an application data sheet (37 CFR 1.76), or the specification must contain, or be amended to contain, such reference in the first sentence following the title. Previously, the Office indicated that if an applicant includes a benefit claim in the application but not in the manner specified by 37 CFR 1.78(a) (e.g., if the claim is included in an oath or declaration or the application transmittal letter) within the time period set forth in 37 CFR 1.78(a), the Office will not require a petition under 37 CFR 1.78(a) and the surcharge under 37 CFR 1.17(t) to correct the claim if the information concerning the claim was recognized by the Office as shown by its inclusion on the filing receipt. If, however, a claim is included elsewhere in the application and not recognized by the Office as shown by its absence on the filing receipt, the Office will require a petition and the surcharge to correct the claim. See Requirements for Claiming the Benefit of Prior-Filed Applications Under Eighteen-Month Publication Provisions, 66 Fed. Reg. 67087, 67089-90 (Dec. 28, 2001). The Office will continue to follow this practice.

Sanctions for Making Improper Benefit Claims

Applicants are simply being advised by this notice that the Office will not recognize any benefit claim where there is no indication of the relationship between the nonprovisional applications, or no indication of the intermediate nonprovisional application that is directly claiming the benefit of a provisional application. Applicants are also reminded that, even if the Office has recognized a benefit claim that includes the proper reference by entering it into the Office's database and including it on applicant's filing receipt, the benefit claim is not a proper benefit claim under 35 U.S.C. 119(e) and/or 35 U.S.C. 120, and 37 CFR 1.78, unless the reference is included in an application data sheet, or the first sentence of the specification, and all other requirements are met.

Part V: Correcting or Adding a Benefit Claim After Filing

The Office will not grant a request for a corrected filing receipt to include a benefit claim unless a proper reference to the prior application(s) is included in the first sentence of the specification, or an application data sheet, within the time period required by 37 CFR 1.78(a). Any request for corrected filing receipt to include a corrected or added benefit claim must be submitted within the time period required by 37 CFR 1.78(a) and be accompanied by an amendment to the specification, or an application data sheet. If the proper reference was previously submitted, a copy of the amendment, the first page of the specification, or the application data sheet, containing the claim should be included with the request for corrected filing receipt. The Office plans to notify applicants on, or with, the

filing receipt that a benefit claim may not have been recognized because it did not include the proper reference. Applicants are advised that only the benefit claims that are listed on the filing receipt have been recognized by the Office. Since the filing receipt and the notification will usually be provided shortly after the filing of the application, applicants should have sufficient opportunity to submit the proper benefit claims within the time period set in 37 CFR 1.78(a) and thus avoid the need to submit a petition under 37 CFR 1.78(a) and the surcharge set forth in 37 CFR 1.17(t). Therefore, applicants should carefully and promptly review their filing receipts in order to avoid the need for a petition and the surcharge.

When an unintentionally delayed benefit claim is submitted with a petition under 37 CFR 1.78(a) and the surcharge set forth in 37 CFR 1.17(t), the benefit claim must include a proper reference to the prior application(s) in order for the petition to be granted. The reference to the prior application(s) must include: (1) the relationship between nonprovisional applications (i.e., continuation, divisional, or continuation-in-part), and (2) the indication of any intermediate application that is directly claiming the benefit of a provisional application, in order to establish copendency throughout the entire chain of prior applications.

Applicants are also reminded that, if an amendment to the specification, or an application data sheet (ADS), is submitted in an application under final rejection, the amendment or ADS must be in compliance with 37 CFR 1.116. The amendment or ADS filed in an application under final rejection will not be entered as a matter of right. See MPEP 714.12 and 714.13. Therefore, applicants should consider filing a request for continued examination (RCE) (including fee and submission) under 37 CFR 1.114 with the petition to accept an unintentionally delayed benefit claim, the surcharge, and an amendment that adds the proper reference to the first sentence of the specification or an ADS.

Part VI: Each Intermediate Prior Application Must Have Proper Reference

If the benefit of more than one prior application is claimed, applicant should also make sure that the proper references are made in each intermediate nonprovisional application in the chain of prior applications. If an applicant desires, for example, the following benefit claim: "This application is a continuation of Application No. C, filed - , which is a continuation of Application No. B, filed - , which claims the benefit of U.S. Provisional Application No. A, filed - ," then Application No. C must include a benefit claim containing a reference to Application No. B and provisional Application No. A, and Application No. B must include a benefit claim containing a reference to provisional Application No. A.

Part VII: Adding an Incorporation-By-Reference Statement in a Benefit Claim is Not Permitted After Filing

An incorporation-by-reference statement added after the filing date of an application is not permitted because no new matter can be added to an application after its filing date. See 35 U.S.C. 132(a). If an incorporation-by-reference statement is included in an amendment to the specification to add a benefit claim after the filing date of the application, the amendment would not be proper. When a benefit claim is submitted after the filing of an application, the reference to the prior application cannot include an incorporation-by-reference statement of the prior application. See Dart Industries v. Banner, 636 F.2d 684,

207 USPQ 273 (C.A.D.C. 1980). Therefore, the Office will not grant a petition to accept a benefit claim that includes an incorporation-by-reference statement of a prior application, unless the incorporation-by-reference statement was submitted on filing of the application.

Inquiries regarding this notice should be directed to Eugenia A. Jones or Joni Y. Chang, Legal Advisors, Office of Patent Legal Administration, by telephone at (703) 305-1622.

February 24, 2003

STEPHEN G. KUNIN
Deputy Commissioner for
Patent Examination Policy

Advances in Brief

Identification of Differentially Expressed Genes in Esophageal Squamous Cell Carcinoma (ESCC) by cDNA Expression Array: Overexpression of *Fra-1, Neogenin, Id-1,* and *CDC25B* Genes in ESCC[1]

Ying Chuan Hu, King Yin Lam, Simon Law, John Wong, and Gopesh Srivastava[2]

Departments of Pathology [Y. C. H., K. Y. L., G. S.] and Surgery [S. L., J. W.], Faculty of Medicine, The University of Hong Kong, Hong Kong

Abstract

Purpose: **This study aims to identify differentially expressed genes in esophageal squamous cell carcinoma (ESCC) through the use of a membrane-based cDNA array.**

Experimental Design: **Two newly established human ESCC cell lines (HKESC-1 and HKESC-2) and one corresponding to a morphologically normal, esophageal epithelium tissue specimen, prospectively collected from the HKESC-2-related patient, were screened in parallel using a cDNA expression array containing gene-specific fragments for 588 human genes spotted onto nylon membranes.**

Results: **The results of cDNA expression array showed that 53 genes were up-regulated 2-fold or higher and 8 genes were down-regulated 2-fold or higher in both ESCC cell lines at the mRNA level. Semiquantitative RT-PCR analysis of a subset of these differentially expressed genes gave results consistent with cDNA array findings. Four of the differentially expressed genes that belong to the categories of oncogenes/tumor suppressor genes (*Fra-1* and *Neogenin*) and cell cycle-related genes (*Id-1* and *CDC25B*) were studied more extensively for their protein expression by immunohistochemistry. The two ESCC cell lines and their corresponding primary tissues, 61 primary ESCC resected specimens and 16 matching, morphologically normal, esophageal epithelium tissues were analyzed. The immunostaining results showed that *Fra-1, Neogenin, Id-1,* and *CDC25B* were overexpressed in both ESCC cell lines and their corresponding primary tumors at the protein level, validating the microarray findings. The results of the clinical specimens showed that the *Fra-1* gene was overexpressed in ESCC compared with normal esophageal epithelium in 53 of 61 cases (87%), *Neogenin* in 57 of 61 cases (93%), *Id-1* in 57 of 61 cases (93%), and *CDC25B* in 48 of 61 cases (79%). Furthermore, the expression of *Fra-1, Neogenin,* and *Id-1* in ESCC correlated with tumor differentiation.**

Conclusions: **Overall, this study demonstrates that multiple genes are differentially expressed in ESCC and provides the first evidence that oncogenes *Fra-1* and *Neogenin* and cell cycle-related genes *Id-1* and *CDC25B* are overexpressed in ESCC.**

Introduction

Esophageal carcinoma is the ninth most common human cancer in the world and the second most common cancer in China (1). In Hong Kong, ESCC[3] accounts for ~90% of esophageal malignant tumors and is the sixth most common cause of cancer death (2). Despite advances in multimodality therapy, the overall 5-year survival rates for ESCCs still remain poor (3). The development of new treatment modalities, diagnostic technologies, and preventive approaches will require a better understanding of the molecular mechanisms underlying esophageal carcinogenesis. Although recent reports have documented alterations of a few oncogenes and tumor suppressor genes in ESCC, the molecular and genetic basis of esophageal carcinogenesis still remains largely unknown (4, 5).

With the emerging technology of cDNA array hybridization, it is now possible to screen for alterations in the expression of many genes simultaneously (6–8). Because the development and progression of cancer are accompanied by complex changes in patterns of gene expression (9, 10), the cDNA array technology provides a very useful tool for studying these complex processes (6). In this study, we used cDNA expression array hybridization to examine the expression of 588 genes in two newly established ESCC cell lines (HKESC-1 and HKESC-2) and one corresponding, morphologically normal, esophageal epithelium tissue specimen collected prospectively from the HKESC-2-related patient. By comparing gene expression levels between normal esophageal epithelium and the ESCC cell lines, we were able to identify the differentially expressed transcripts in ESCC. Subsequent semiquantitative RT-PCR analyses vali-

Received 3/20/01; revised 5/7/01; accepted 5/8/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Presented in part at the 91st Annual Meeting of the American Association for Cancer Research, Inc. San Francisco, CA, 2000.

[2] To whom requests for reprints should be addressed, at Department of Pathology, University of Hong Kong, University Pathology Building, Queen Mary Hospital, Pok Fu Lam Road, Hong Kong. Phone: (852) 2855-4859; Fax: (852) 2872-5197; E-mail: gopesh@pathology.hku.hk.

[3] The abbreviations used are: ESCC, esophageal squamous cell carcinoma; *Fra-1,* fos-related antigen 1; *CDC25B,* cell division cycle 25B; *Id-1,* inhibitor of differentiation 1 (inhibitor of DNA binding 1); IH, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; *FPR1,* formyl peptide receptor 1; *RANTES,* regulated on activation, normal T expressed, and secreted; AP-1, activator protein-1; CDK, cyclin-dependent kinase; DAB, 3,3′-diaminobenzidine.

dated the cDNA array results. Expression of the protein products of four of these differentially expressed genes that belong to the categories of oncogenes/tumor suppressor genes (*Fra-1* and *Neogenin*) and cell cycle-related genes (*Id-1* and *CDC25B*) was further evaluated by IH in a large series of ESCC tumor specimens.

Materials and Methods

Cell Culture and Tissue Specimen. Two human ESCC cell lines, HKESC-1 and HKESC-2, have been established recently in our laboratory. HKESC-1 has been reported previously (11). Both cell lines were from Hong Kong Chinese patients with moderately differentiated ESCC; HKESC-1 was from a 47-year-old man, whereas HKESC-2 was from a 46-year-old woman. The squamous epithelial nature of HKESC-2 was confirmed by both electron microscopy (presence of tonofilaments and desmosomes) and immunohistochemical staining (positive for cytokeratins; data not shown). Both cell lines grew as adherent monolayers (11). HKESC-2 was maintained in the same conditions as HKESC-1 (11). Cells were harvested from passage 31 of HKESC-1 and passage 4 of HKESC-2 at 80–90% confluency, respectively.

One morphologically normal, esophageal epithelium tissue specimen, collected prospectively from the HKESC-2-related patient, was used as a control for the array experiment. For obtaining high-purity normal esophageal epithelium tissue specimen, the morphologically normal esophageal epithelium at least 5 cm away from the tumor margin was carefully dissected out from other tissues of the freshly resected esophagectomy specimen from the HKESC-2-related patient and evaluated microscopically. Unfortunately, the collected normal esophageal epithelium tissue from the HKESC-1-related patient could not be used as a control because the specimen was too small and only a small amount of RNA could be extracted from it.

cDNA Arrays, Probes, Hybridization, and Data Analysis. Atlas Human cDNA Expression Array membranes used in this study were purchased from Clontech (Palo Alto, CA). The membrane contained 10 ng of each gene-specific cDNA from 588 known genes and 9 housekeeping gene fragments (Fig. 1). Several plasmid and bacteriophage DNAs and blank spots are also included as negative and blank controls to confirm hybridization specificity.[4]

Total RNA was extracted using the Trizol reagent protocol (Life Technologies, Inc., Gaithersburg, MD) from the two ESCC cell lines (HKESC-1 and HKESC-2) and one corresponding, morphologically normal esophageal epithelium collected prospectively from the HKESC-2-related patient. mRNA was then isolated from the total RNA using the Straight A's mRNA Isolation System (Novagen, Madison, WI). The ^{32}P-labeled cDNA probes were generated by reverse transcription of 1 μg of mRNA of each sample in the presence of [α-^{32}P]dATP. Equal amounts of cDNA probes (3×10^6 cpm/μl) from the ESCC cell lines and normal esophageal epithelium were then hybridized to separate Atlas Human cDNA array membranes for 24 h at 42°C and washed according to the supplier's instructions. The array membranes were then exposed to X-ray film at −70°C for 2–5 days. Autoradiographic intensity was analyzed using AtlasImage analysis software (version 1.01; Clontech). The signal intensities were normalized by comparing the expression of housekeeping genes *GAPDH* (G12) and *HLA-C* (G14):

$$\text{Intensity ratio} = \frac{\text{Adjusted intensity on array} - \text{HKESC-1 or HKESC} - 2}{\text{Adjusted intensity on array} - \text{normal}} \times \text{Normalization coefficient}$$

$$\text{Adjusted intensity} = \text{Intensity} - \text{Background}$$

$$\text{Normalization coefficient} = \left[\left(\frac{\text{Adjusted intensity } \textit{GAPDH} \text{ on array} - \text{normal}}{\text{Adjusted intensity } \textit{GAPDH} \text{ on array} - \text{HKESC-1 or HKESC-2}}\right)\left(\frac{\text{Adjusted intensity HLA} - \text{Con array} - \text{normal}}{\text{Adjusted intensity HLA} - \text{Con array} - \text{HKESC-1 or HKESC-2}}\right)\right] \div 2$$

Genes were considered to be up-regulated when the intensity ratio between expression in the ESCC cell lines compared with normal esophageal epithelium was 2-fold or greater. Genes were labeled as down-regulated when the ratio between normal and ESCC cell lines was 2-fold or higher.

RT-PCR. cDNA was generated using 1 μg of total RNA from the two ESCC cell lines (HKESC-1 and HKESC-2) and one corresponding, morphologically normal, esophageal epithelium collected prospectively from the HKESC-2-related patient as template and 2.5 mM Oligo $d(T)_{16}$ primers in a 20-μl reaction mixture, and the reverse transcription was carried out at 42°C for 1 h, followed by 95°C for 10 min using the GeneAmp RNA PCR Core kit (Perkin-Elmer, Branchburg, NJ). Two μl of cDNA were amplified in a 25-μl PCR reaction mixture containing 1× PCR buffer, 1.9 or 2.4 or 2.9 mM $MgCl_2$, 0.5 μM primers, 0.18 mM deoxynucleotides triphosphates, 1 unit of AmpliTaq Gold DNA polymerase with hot-start PCR as follows: 95°C for 10 min, followed by 25–35 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C (for primers of *cyclin D1, Id-1, CDC25B, FPR1, RANTES,* and *GAPDH*) or 62°C (for primers of *Fra-1*) or 65°C (for primers of *Neogenin*), 1 min extension at 72°C. Finally, PCR products were fully extended by incubating at 72°C for 10 min. The PCR reagents were purchased from Perkin-Elmer.

The sequences of gene specific primers for RT-PCR were the same as those of cDNA array (data not shown because of the copyright agreement by Clontech, Palo Alto, CA), except for the primers specific for *Fra-1*, which were the same as described before (12). All of the primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The cycle number was optimized for each gene-specific primer pair to ensure that amplification was in the linear range and the results were semiquantitative. Twelve μl of PCR product were visualized by

[4] A complete list of the 588 genes with array positions and GenBank accession numbers of the Atlas Human Expression Array used here can also be accessed through the web site http://www.clontech.com/clontech/APR97UPD/Atlaslist.html.

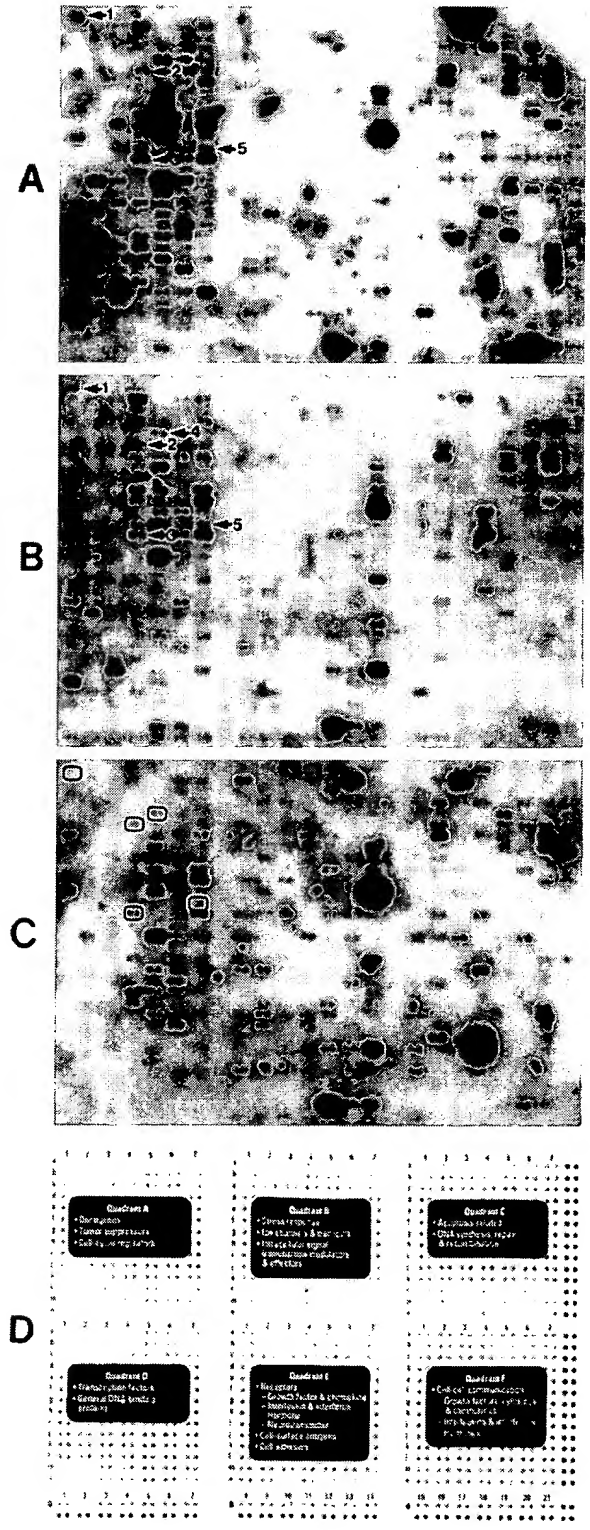

Fig. 1 A–C, gene expression profiles of two human ESCC cell lines HKESC-1 (*A*) and HKESC-2 (*B*) and one morphologically normal esophageal epithelium (*C*) from the HKESC-2-related patient using the Atlas Human cDNA Expression Array. Some of the differentially expressed genes are indicated: *1*, c-*myc* (A1a); *2*, *Fra-1* (A4f); *3*, *Neogenin* (A4n); *4*, *Id-1* (A5e); and *5*, *CDC25B* (A7m). *D*, schematic diagram of Atlas Human cDNA Expression Array. The array contains 588 human genes spotted in duplicate and divided into six functional categories (*Quadrants A–F*). Three blank (G1, G8, and G15) and nine negative (G2–4, G9–11, and G16–18) controls are included to confirm hybridization specificity. Nine housekeeping genes (G5–7, G12–14, and G19–21) are also included in the array for normalizing mRNA abundance. Genomic DNA spots (*dark dots*) serve as orientation marks to facilitate in the determination of the coordinates of hybridization signals. A complete gene list with array coordinates and GenBank accession numbers is available at world wide web site http://www.clontech.com/clontech/APR97 UPD/Atlaslist.html.

electrophoresis on a 2% agarose gel stained with ethidium bromide and quantitated by densitometry using a dual-intensity transilluminator equipped with Gelworks 1D Intermediate software (version 2.51).

Collection of Tissues and Clinicopathological Data. The tissues were obtained from 61 (50 men and 11 women) patients with ESCC resected between 1996 and 1998 in Queen Mary Hospital, The University of Hong Kong. The patients' ages ranged from 41 to 83 years, with a mean age of 65 years. The specimens were dissected and examined in the fresh state. Representative tissue specimens from tumors and matching normal esophageal epithelium tissues were snap-frozen in liquid nitrogen and stored at −80°C. Other representative blocks were taken and processed in paraffin for histological examination. The carcinomas were found in the upper (n = 10; 16%), middle (n = 35; 57%), and lower (n = 16; 26%) third of the esophagus. The median length of the tumors was 5.5 cm (range, 1–11). The histology of the carcinomas was reviewed according to the criteria described previously (13). The ESCC tumors were well differentiated in 20 (33%) cases, moderately differentiated in 29 (48%), and poorly differentiated in 12 (20%). The carcinomas were staged according to the Tumor-Node-Metastasis classification (14). Many tumors were stage III (n = 35, 57%) or II (n = 23, 38%); of the remainder, one was stage I and two were stage IV.

Immunohistochemistry. Expression of *Fra-1*, *Neogenin*, *Id-1*, and *CDC25B* was investigated by the streptavidin-biotin-peroxidase complex method. Briefly, 6-μm frozen sections were cut from two pellets harvested from cultured cell lines HKESC-1 and HKESC-2, the cell lines' corresponding primary tissues, 61 primary ESCC tumors, and 16 matching, morphologically normal, esophageal epithelium specimens. After endogenous peroxidase activity was quenched and nonspecific binding was blocked, polyclonal rabbit anti-Fra-1, goat anti-Neogenin, rabbit anti-Id-1, and goat anti-CDC25B antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated at 4°C overnight at a dilution of 1:100 for *Fra-1*, 1:40 for *Neogenin* and *Id-1*, and 1:30 for *CDC25B*, respectively. The secondary antibody was biotinylated swine anti-rabbit (for *Fra-1* and *Id-1*) or rabbit anti-goat (for *Neogenin* and *CDC25B*) antibody (DAKO, Glostrup, Denmark) used at a dilution of 1:200 for 30 min at 37°C. After washing, sections were incubated with StreptABComplex/horseradish peroxidase (DAKO; 1:100 dilution) for 30 min at 37°C. Negative controls were performed by replacing the primary antibody by normal serum.

Each section was independently assessed by two histopathologists without prior knowledge of the patients' other data. Scoring was based on the percentage of positive cells. The staining was identified as: −, no expression; +, <10% of cells were stained; ++, 10–50% of cells stained; and +++, >50% of cells stained. From ++ to +++ was defined as overexpression.

Statistical Analysis. Comparisons between groups were performed using the χ^2 test and t test when appropriate. $P < 0.05$ was used to determine statistical significance. All statistical tests were performed with the GraphPad Prism software version 3.0 (GraphPad Software, Inc., San Diego, CA).

Results and Discussion

In this study, we first used cDNA expression array hybridization to identify genes that were differentially expressed in ESCC compared with normal esophageal epithelium. Two newly established ESCC cell lines in our laboratory were selected for cDNA array analysis to assure large quantities of high-purity tumor mRNA. The comparison of the autoradiographic intensities between ESCC cell lines and normal esophageal epithelium showed that 65 and 59 genes were up-regulated 2-fold or higher and 11 and 21 genes were down-regulated 2-fold or more in HKESC-1 and HKESC-2, respectively. Among these differentially expressed genes, 53 genes were up-regulated and 8 genes were down-regulated in both cell lines (Fig. 1 and Table 1). No signals were visible in the three blank spots (G1, G8, and G15) and nine negative control spots (G2–4, G9–11, and G16–18; Fig. 1), indicating that the cDNA array hybridization was highly specific. Among the 61 differentially expressed genes in both cell lines, 49 genes such as *Fra-1, Neogenin, Id-1,* and *CDC25B* genes were identified as differentially expressed in ESCC for the first time; 12 other differentially expressed genes have been described to be overexpressed in ESCC previously. The genes overexpressed in both of the ESCC cell lines belong to the categories of oncogenes/tumor suppressor genes, cell cycle-related genes, genes for DNA synthesis, DNA binding genes, or apoptosis-related genes (Table 1). The 8 genes that were down-regulated in both ESCC cell lines comprised genes for signal transduction (*guanine nucleotide regulatory protein NET1, protein kinase C-β II, cAMP-dependent protein kinase catalytic d-subunit and EPLG3*), genes for signaling proteins (*RANTES protein T-cell specific, Somatomedin A,* and *FPR1*), and the gene for MAL protein. These findings demonstrated that multiple genes are differentially expressed in ESCC at mRNA level.

To further validate the cDNA array approach, we performed semiquantitative RT-PCR to analyze the expression levels of 8 genes, *cyclin D1, Fra-1, Neogenin, Id-1, CDC25B, FPR1, RANTES,* and *GAPDH.* The results of RT-PCR analysis (Fig. 2) were consistent with the expression profiles obtained through cDNA array hybridization (Fig. 1).

Genes that belong to the categories of oncogenes/tumor suppressor genes and cell cycle-related genes are often implicated in the pathogenesis of various cancers (4, 5, 15). Significantly, a number of the differentially expressed genes identified by cDNA array hybridization in both ESCC cell lines belong to these categories. Four of these differentially expressed genes that were identified for the first time to be overexpressed in ESCC in this study, the oncogenes *Fra-1* and *Neogenin* and the cell cycle related genes *Id-1* and *CDC25B,* were selected for more detailed study for their protein expression in a large series of ESCC tumor specimens by IH. Moreover, these genes have been reported to be overexpressed in other tumor cell lines or primary tumors (16–24). The other consideration for selecting these particular genes for more extensive study was that the suitable antibodies of these genes were commercially available for the IH studies. The protein expression of these four genes was investigated in the two ESCC cell lines and their corresponding primary tissues, 61 primary ESCC tumors, and 16 matching, morphologically normal, esophageal epithelium specimens. The results of immunostaining are summarized in Tables 2 and 3 and are shown in Fig. 3. The protein products of *Fra-1, Neogenin, Id-1,* and *CDC25B* genes were found to be overexpressed in both the ESCC cell lines and their corresponding primary tumors (Table 2), validating the cDNA array results.

Fra-1 is one component of the AP-1 complex (25). The AP-1 components are considered to play key roles in signal transduction pathways involved in complex cellular growth, differentiation, and tumorigenesis (16). Previous studies indicated that increased AP-1 activity is a necessary event in the transformation of mouse epidermal cells (26, 27). *Fra-1* overexpression has been found in kidney and thyroid cancer (16, 17). These observations suggest that *Fra-1* overexpression might play an important role in malignant transformation of epithelial cells. In the present work, *Fra-1* mRNA overexpression was detected in both ESCC cell lines by cDNA array analysis (Fig. 1) and RT-PCR (Fig. 2*B*). Also, the majority of ESCC tumors (53 of 61, 87%; Table 3; Fig. 3*B*) had enhanced expression of *Fra-1.* Fra-1 protein expression was localized in the nuclei of ESCC tumor cells (Fig. 3*B*). In contrast, morphologically normal, esophageal epithelium tissues showed low expression of Fra-1. The expression of Fra-1 was often focal in morphologically normal esophageal epithelium and always restricted to the basal cell layer (Fig. 3*A*). The well or moderately differentiated ESCC showed more intense expression of Fra-1 than poorly differentiated ones ($P < 0.0001$; Table 3).

Neogenin encodes a 1461-amino acid protein with 50% amino acid identity to *DCC* (*deleted in colorectal cancer*; Ref. 18). It has been suggested to play an integral role in regulating differentiation and/or cell migration events within many embryonic and adult tissues (28). *Neogenin* expression has been detected at low levels in many adult tissues but not including esophagus (18). Overexpression of *Neogenin* has been observed in a wide variety of human cancer cell lines from cancers of breast, pancreas, brain, cervix, colon, and rectum (18). However, there is no information about the status of *Neogenin* expression in human primary cancers including esophageal cancer. In the current study, our cDNA array (Fig. 1) and RT-PCR (Fig. 2*B*) results showed that *Neogenin* mRNA was overexpressed in both ESCC cell lines. Neogenin protein overexpression was noted in 93% (57 of 61) of ESCCs (Table 3; Fig. 3*D*). The expression was localized in the cytoplasm of tumor cells (Fig. 3*D*). In contrast, the expression of Neogenin protein in morphologically normal esophageal epithelium was negative or negligible and was restricted to the highly proliferative basal cells (Fig. 3*C*). The well or moderately differentiated ESCC

Table 1 List of differentially expressed genes in the ESCC cell lines HKESC-1 and HKESC-2 when compared with one corresponding, morphologically normal, esophageal epithelium tissue specimen (N) from the HKESC-2-related patient using cDNA expression array

Location	Name of gene	Intensity Ratio HKESC-1/N	Intensity Ratio HKESC-2/N
Genes up-regulated in both ESCC cell lines			
Oncogene/Tumor suppressor genes			
A1a	*c-myc*	2.5	3.0
A2b	*IGFBP-2*	6.9	6.0
A3b	*Snon*	7.2	6.0
A3i	*rhoA* (MDR protein)	2.8	3.9
A3k	*DCC*	5.1	3
A4b	*APC*	2.6	3.9
A4c	*BRCA2*	30029/0	42730/0
A4f	*Fra-1*	10.8	7.8
A4g	*Ezrin*	21936/0	36475/0
A4h	*JUN-D*	35.4	60.6
A4j	*PEP1*	3.6	4.8
A4k	*EB1*	2.6	3.0
A4l	*C-CBL*	2.7	2.2
A4m	*Smad1*	3.2	3.0
A4n	*Neogenin*	2.4	3.2
Cell cycle-related genes			
A5e	*Id-1*	3.4	3.7
A5g	*P58/GTA1*	4.0	2.2
A6g	*Cyclin D1*	2.1	2.3
A6l	*Cyclin B1*	2.4	3.2
A6m	*Cyclin E*	3.5	3.3
A7b	*Cyclin G2*	2.4	3.1
A7d	*p35*	6.9	7.2
A7l	*C-1*	2.6	2.6
A7m	*CDC25B*	2.0	2.5
Apoptosis-associated genes			
C1i	*Adenosine A1 receptor*	22.2	5.9
C4l	*Apopain*	8.7	13.7
Genes for DNA synthesis/repair/recombination proteins			
C6d	*XRCC1*	3.2	2.9
C6l	*DNA Topoisomerase II*	14759/0	22201/0
C7n	*Dnase X*	5.1	4.4
Genes for DNA binding/transcription factors			
D1c	*CCAT-binding protein*	9.9	8.8
D1d	*Id-3*	6.5	6.6
D1e	*BTEB2*	7.4	9.0
D1g	*Id-2*	9.8	7.6
D1l	*TAX*	3.0	4.4
D1n	*CNBP*	4.3	2.1
D2a	*CCAAT displacement protein*	4.2	4.5
D2c	*APRF*	33987/0	29011/0
D2d	*hSNF2b*	42.2	40.7
D2f	*TAXREB67*	39440/0	49383/0
D2i	*TCF5*	37478/0	23080/0
D3a	*hSNF2a*	7.1	4.2
D3b	*DB1*	316.5	225.0
D3c	*D-binding protein*	37796/0	21621/0
D3g	*PAX-8*	13.6	7.2
D3j	*P15 subunit*	38725/0	40864/0
D3k	*Guanine nucleotide-binding protein G-S*	33.0	49.2
D4c	*AP-2*	7.1	4.9
D4j	*NF-E1*	34506/0	15924/0
D5k	*PAX3*	24.8	10.6
D7k	*TAFII31*	7.0	4.8
Genes for signal proteins			
F5a	*NGF-2*	16.7	4.6
F5b	*MIP2α*	39223/0	19827/0
F5f	*IL-8*	39239/0	11638/0
Genes down-regulated in both ESCC cell lines			
Gene for iron channel/transport protein			
B1b	*MAL protein*	1/3.3	0/11989
Genes for signal transduction			
B4g	*Guanine nucleotide regulatory protein NET1*	0/28564	1/23.7
B5j	*Protein kinase c-β II*	0/29588	0/29588
B6b	*cAMP-dependent protein kinase α-subunit*	0/41060	1/304
B6n	*EPLG3*	0/22304	1/2.5
Genes for cell signaling proteins			
F1a	*Somatomedin A*	0/19560	0/19560
F1k	*FMLP-related receptor I*	0/29220	0/29220
F2j	*RANTES protein T-cell specific*	0/36868	1/3.2

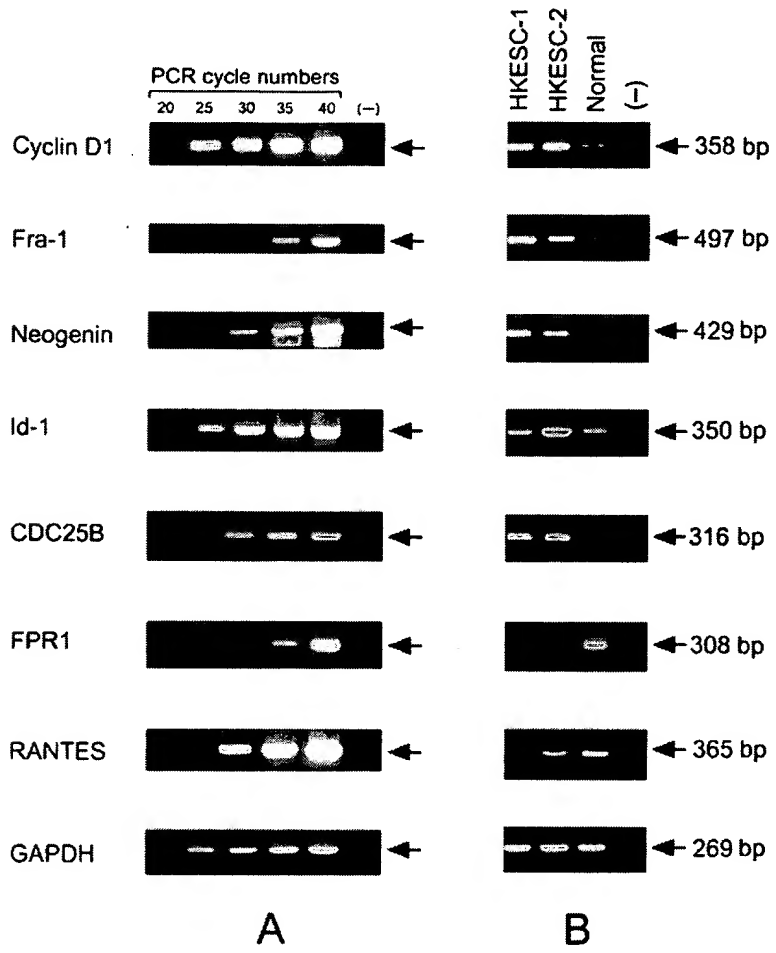

Fig. 2 RT-PCR analysis of *cyclin D1, Fra-1, Neogenin, Id-1, CDC25B, FPR1, RANTES,* and *GAPDH* genes in ESCC cell lines HKESC-1 and HKESC-2 and one corresponding, morphologically normal epithelium (*Normal*) from the HKESC-2-related patient. *A*, determination of optimal number of PCR cycles for different gene-specific primer pairs. mRNA from HKESC-1 was used to determine the optimal number of PCR cycles for genes *cyclin D1, Fra-1, Neogenin, Id-1, CDC25B,* and *GAPDH*. mRNA from the normal esophageal epithelium was used to determine the optimal number of PCR cycles for genes *FPR1* and *RANTES*. *B*, expression of *cyclin D1* (25 cycles), *Fra-1* (32 cycles), *Neogenin* (30 cycles), *Id-1* (25 cycles), *CDC25B* (28 cycles), *FPR1* (35 cycles), *RANTES* (28 cycles), and *GAPDH* (25 cycles) genes in two ESCC cell lines HKESC-1 and HKESC-2 and one corresponding, morphologically normal esophageal epithelium (*Normal*) from the HKESC-2-related patient.

Table 2 Summary of immunohistochemical staining results in ESCC cell lines and their corresponding primary tissue specimens

	Cell lines		Primary tissues[a]			
	HKESC-1	HKESC-2	T_1	N_1	T_2	N_2
Fra-1	+++[b]	+++	+++	+	++	+
Neogenin	+++	+++	+++	−	++	+
Id-1	+++	+++	+++	++	++	−
CDC25B	+++	+++	+++	−	++	+

[a] T, ESCC tumor; N, morphologically normal esophageal epithelium.
[b] Expression: −, no expression; +, <10% cells positive; ++, ≥10% and <50% cells positive; +++, ≥50% cells positive. ++ to +++ was considered as overexpression.

showed more intense expression of *Neogenin* than poorly differentiated ones ($P = 0.0047$; Table 3).

Id-1 is a cell cycle-related gene that encodes a helix-loop-helix protein. *Id-1* plays an important role not only in suppressing cellular differentiation but also in enhancing cellular proliferation (29, 30). Generally, Id-1 protein is highly expressed in growing cells, and its expression is down-regulated upon differentiation in many cell types. Although Id-1 is expressed in a variety of fetal tissues and overexpressed in tumors from brain and lung (19), its expression in ESCC is unknown. In this study, we observed that *Id-1* mRNA was overexpressed in both ESCC cell lines (Figs. 1 and 2*B*). Also, Id-1 protein overexpression was frequent in human primary ESCC tumors (57 of 61, 93%; Table 3; Fig. 3*F*). The Id-1 protein was localized in the cytoplasm of tumor cells (Fig. 3*F*). In contrast, the expression of Id-1 protein in morphologically normal esophageal epithelium was either negative or negligible and was restricted to the basal and parabasal cells (Fig. 3*E*). The well or moderately differentiated ESCC showed more intense expression of *Id-1* than poorly differentiated ones ($P = 0.0156$; Table 3).

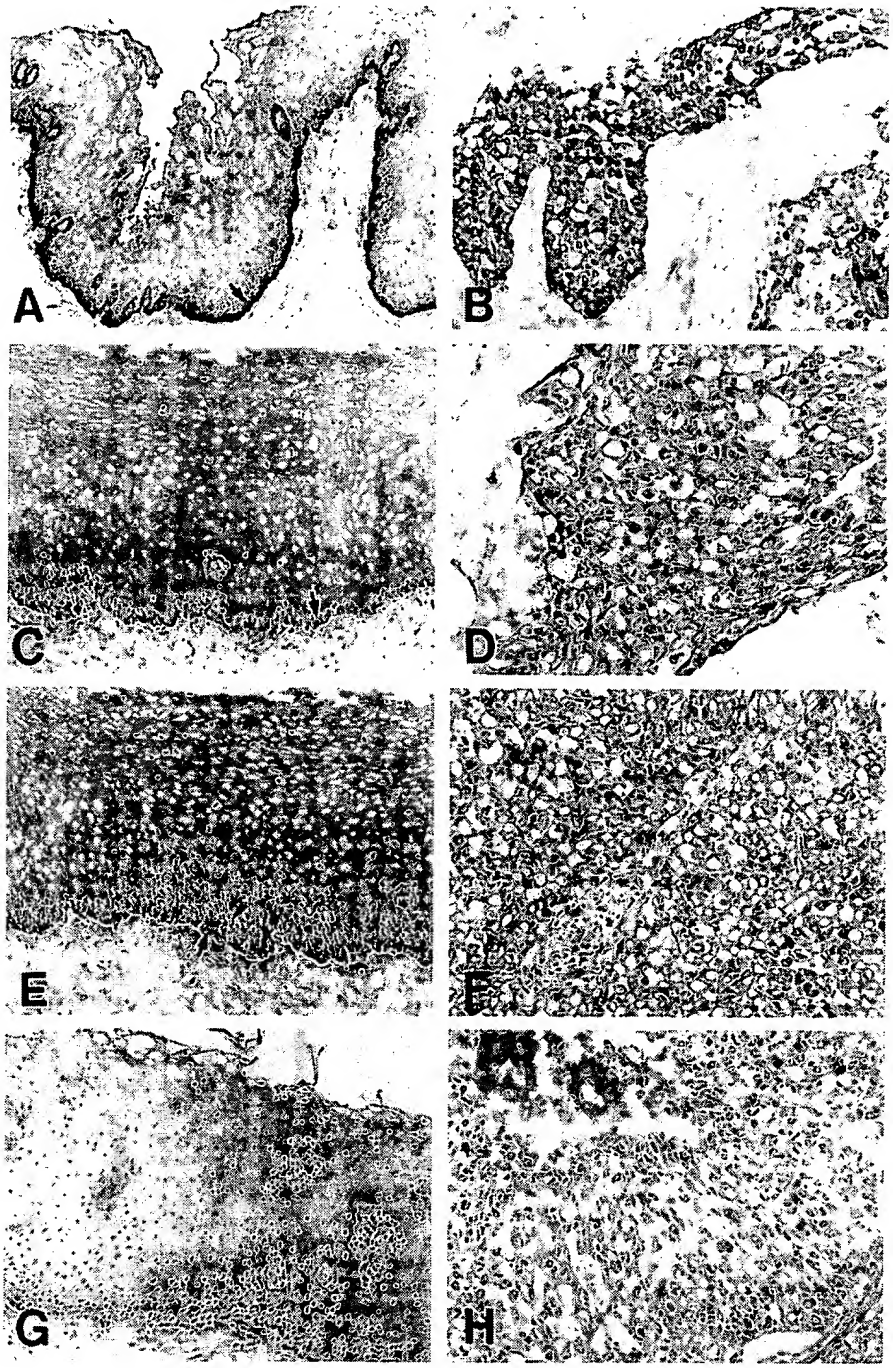

Fig. 3 Photomicrographs of *Fra-1, neogenin, Id-1,* and *CDC25B* expression by IH in morphologically normal esophageal epithelium and ESCC. *A, Fra-1* IH in morphologically normal esophageal epithelium showing that *Fra-1* expression was restricted to the basal cell layer (*arrow*); DAB ×160. *B, Fra-1* IH in ESCC showing that the nuclei of tumor cells are strongly positive for *Fra-1*; DAB × 400. *C, Neogenin* IH in morphologically normal esophageal epithelium showing that *Neogenin* expression was restricted to the basal cell layer (*arrow*); DAB ×330. *D, Neogenin* IH in ESCC showing *Neogenin* expression in the cytoplasm of tumor cells; DAB ×500. *E, Id-1* IH in morphologically normal esophageal epithelium showing *Id-1* expression was restricted to the basal (*arrow*) and parabasal (*arrowhead*) cell layers; DAB ×330. *F, Id-1* IH in ESCC showing Id-1 expression in the cytoplasm of tumor cells; DAB ×500. *G, CDC25B* IH in morphologically normal esophageal epithelium showing lack of immunoreactivity; DAB × 330. *H, CDC25B* IH in ESCC showing *CDC25B* expression in the nuclei of most tumor cells; DAB ×500.

CDC25B is a cell cycle-related gene. Its product is a phosphatase that catalyzes the removal of inhibitory phosphate from the CDK family of proteins (31). *CDC25B* can dephosphorylate threonine 14, tyrosine 15, or both on CDKs and activate cyclin/CDK complexes to stimulate cell proliferation (32). *In vitro* transforming experiments have demonstrated that *CDC25B* is also a potential oncogene (20). Overexpression of *CDC25B* has been found in cancers arising from breast (20), stomach (21), lung (22), and head and neck (23), and in non-Hodgkin's lymphoma (24). In this study, we demonstrated that the mRNA of *CDC25B* was highly expressed in both ESCC cell lines by cDNA array (Fig. 1) and RT-PCR (Fig. 2*B*). Furthermore, *CDC25B* was overexpressed in 79% (48 of 61) primary ESCC tumors by IH (Table 3; Fig. 3*H*). CDC25B protein

Table 3 Summary of IH staining results in clinical ESCC tumors and normal esophageal epithelium tissues

	Fra-1					Neogenin					Id-1					CDC25B				
Diagnosis	−[a]	+	++	+++	*P*	−	+	++	+++	*P*	−	+	++	+++	*P*	−	+	++	+++	*P*
Normal (n = 16)	1	15	0	0	<0.0001	6	10	0	0	<0.0001	3	12	1	0	<0.0001	9	7	0	0	<0.0001
Carcinoma (n = 61)	4	4	22	31		3	1	7	50		3	1	8	49		10	3	19	29	
Well (n = 20)	0	0	9	11		1	0	2	17		0	1	3	16		2	2	7	9	
Moderate (n = 29)	1	0	11	17	<0.0001	0	0	1	28	0.0047	0	0	3	26	0.0156	6	1	10	12	0.5720
Poor (n = 12)	3	4	2	3		2	1	4	5		3	0	2	7		2	0	2	8	

[a] Expression: −, no expression; +, <10% cells positive; ++, ≥10% and <50% cells positive; +++, ≥50% cells positive. ++ to +++ was considered as overexpression.

expression was localized mainly in the nuclei of tumor cells (Fig. 3*H*). On the other hand, the expression of *CDC25B* in morphologically normal esophageal epithelium tissues was either negative or very weak (Fig. 3*G*). In the case of *CDC25B*, there was no correlation between gene expression and ESCC differentiation (P = 0.5720; Table 3).

In summary, all four of the genes selected for further study demonstrated a significantly higher incidence of overexpression in primary ESCCs than morphologically normal esophageal epithelium tissues ($P < 0.0001$; Table 3). Furthermore, three of them, *Fra-1, Neogenin,* and *Id-1* were more highly expressed in tumors with greater differentiation. *CDC25B* did not demonstrate this correlation (Table 3). The expression of these genes did not correlate to age at presentation or gender of patients or tumor site, size, or stage. The differentiation of squamous cell carcinoma bears no relationship with the stage of the tumor (4). In this study, the expression of *Fra-1, Neogenin,* and *Id-1* was more often noted in the well/moderately differentiated squamous cell carcinoma. This is consistent with the theory that poorly differentiated squamous cell carcinoma arises at the early stage of carcinogenesis. In the later stages of tumor progression, the squamous cell carcinoma becomes more mature in appearance (well/moderately differentiated).

Unfortunately, the complete follow-up data were available only for some of these patients. Nevertheless, all these patients died within 2 years of resection of the primary tumors. Also, the *Fra-1, Neogenin, Id-1,* and *CDC25B* were highly expressed in squamous cell carcinomas. Thus, it is unlikely that the expression of these genes acts as an independent prognostic factor in these tumors.

Overall, our data demonstrate that multiple genes are differentially expressed in ESCC and show for the first time that oncogenes *Fra-1* and *Neogenin* and cell cycle-related genes *Id-1* and *CDC25B* are overexpressed in ESCC. Additional studies are required to determine the roles of these and other differentially expressed genes in the molecular pathogenesis of ESCC.

References

1. Blot, W. J. Esophageal cancer trends and risk factors. Semin. Oncol., *21:* 403–410, 1994.

2. Lam, K. Y., and Ma, L. Pathology of esophageal cancers: local experience and current insights. Chin. Med. J., *10:* 459–464, 1997.

3. Law, S. Y., Fok, M., Cheng, S. W., and Wong, J. A comparison of outcome after resection for squamous cell carcinomas and adenocarcinomas of the esophagus and cardia. Surg. Gynecol. Obstet., *175:* 107–112, 1992.

4. Lam, K. Y., Tsao, S. W., Zhang, D., Law, S., He, D., Ma, L., and Wong, J. Prevalence and predictive value of p53 mutation in patients with esophageal squamous cell carcinomas: a prospective clinico-pathological study and survival analysis of 70 patients. Int. J. Cancer, *74:* 212–219, 1997.

5. Montesano, R., Hollstein, M., and Hainaut, P. Genetic alterations in esophageal cancer and their relevance to etiology and pathogenesis: a review. Int. J. Cancer, *69:* 225–235, 1996.

6. DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, Y., Su, Y. A., and Trent, J. M. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat. Genet., *14:* 457–460, 1996.

7. Fuller, G. N., Rhee, C. H., Hess, K. R., Caskey, L. S., Wang, R., Bruner, J. M., Yung, W. K. A., and Zhang, W. Reactivation of insulin-like growth factor binding protein 2 expression in glioblastoma multiforme: a revelation by parallel gene expression profiling. Cancer Res., *59:* 4228–4232, 1999.

8. Shim, C., Zhang, W., Rhee, C. H., and Lee, J. H. Profiling of differentially expressed genes in human primary cervical cancer by complementary DNA expression array. Clin. Cancer Res., *4:* 3045–3050, 1998.

9. Vogelstein, B., and Kinzler, K. W. The multistep nature of cancer. Trends Genet., *9:* 138–141, 1993.

10. Zhang, L., Zhou, W., Velculescu, V. E., Kern, S. E., Hruban, R. H., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Gene expression profiles in normal and cancer cells. Science (Wash. DC), *276:* 1268–1272, 1997.

11. Hu, Y. C., Lam, K. Y., Wan, T. S. K., Fang, W. G., Ma, E. S. K., Chan, L. C., and Srivastava, G. Establishment and characterization of HKESC-1, a new cancer cell line from human esophageal squamous cell carcinoma. Cancer Genet. Cytogenet., *118:* 112–120, 2000.

12. Risse-Hackl, G., Adamkiewicz, J., Wimmel, A., and Schuermann, M. Transition from SCLC to NSCLC phenotype is accompanied by an increased TRE-binding activity and recruitment of specific AP-1 proteins. Oncogene, *16:* 3057–3068, 1998.

13. Lam, K. Y., He, D., Ma, L., Zhang, D., Ngan, H. Y., Wan, T. S., and Tsao, S. W. Presence of human papillomavirus in esophageal squamous cell carcinomas of Hong Kong Chinese and its relationship with *p53* gene mutation. Hum. Pathol., *28:* 657–663, 1997.

14. Beahrs, O. H., Henson, D. E., Hutter, R. V. P., and Kennedy, B. J. (eds.). Manual for Staging of Cancer, Ed. 4, pp. 57–59. Philadelphia: J. B. Lippincott Co., 1992.

15. Hunter, T. Oncoprotein networks. Cell, *88:* 333–346, 1997.

16. Battista, S., de Nigris, F., Fedele, M., Chiappetta, G., Scala, S., Vallone, D., Pierantoni, G. M., Megar, T., Santoro, M., Viglietto, G., Verde, P., and Fusco, A. Increase in AP-1 activity is a general event in thyroid cell transformation *in vitro* and *in vivo*. Oncogene, *17:* 377–385, 1998.

17. Urakami, S., Tsuchiya, H., Orimoto, K., Kobayashi, T., Igawa, M., and Hino, O. Overexpression of members of the AP-1 transcriptional factor family from an early stage of renal carcinogenesis and inhibition of cell growth by *AP-1* gene antisense oligonucleotides in the *Tsc2* gene

mutant (Eker) rat model. Biochem. Biophys. Res. Commun., *241:* 24–30, 1997.

18. Meyerhardt, J. A., Look, A. T., Bigner, S. H., and Fearon, E. R. Identification and characterization of *Neogenin*, a DCC-related gene. Oncogene, *14:* 1129–1136, 1997.

19. Zhu, W., Dahmen, J., Bulfone, A., Rigolet, M., Hernandez, M. C., Kuo, W. L., Puelles, L., Rubenstein, J. L., and Israel, M. A. *Id* gene expression during development and molecular cloning of the human *Id-1* gene. Brain Res. Mol. Brain Res., *30:* 312–326, 1995.

20. Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M., and Beach, D. CDC25 phosphatases as potential human oncogenes. Science (Wash. DC), *269:* 1575–1577, 1995.

21. Kudo, Y., Yasui, W., Ue, T., Yamamoto, S., Yokozaki, H., Nikai, H., and Tahara, E. Overexpression of cyclin-dependent kinase-activating CDC25B phosphatase in human gastric carcinomas. Jpn. J. Cancer Res., *88:* 947–952, 1997.

22. Wu, W., Fan, Y. H., Kemp, B. L., Walsh, G., and Mao, L. Overexpression of cdc25A and cdc25B is frequent in primary non-small cell lung cancer but is not associated with overexpression of c-myc. Cancer Res., *58:* 4082–4085, 1998.

23. Gasparotto, D., Maestro, R., Piccinin, S., Vukosavljevic, T., Barzan, L., Sulfaro, S., and Boiocchi, M. Overexpression of CDC25A and CDC25B in head and neck cancers. Cancer Res., *57:* 2366–2368, 1997.

24. Hernandez, S., Hernandez, L., Bea, S., Cazorla, M., Fernandez, P. L., Nadal, A., Muntane, J., Mallofre, C., Montserrat, E., Cardesa, A., and Campo, E. cdc25 cell cycle-activating phosphatases and c-myc expression in human non-Hodgkin's lymphomas. Cancer Res., *58:* 1762–1767, 1998.

25. Cohen, D. R., and Curran, T. *Fra-1:* a serum-inducible, cellular immediate-early gene that encodes a fos-related antigen. Mol. Cell. Biol., *8:* 2063–2069, 1988.

26. Li, J. J., Dong, Z., Dawson, M. I., and Colburn, N. H. Inhibition of tumor promoter-induced transformation by retinoids that transrepress AP-1 without transactivating retinoic acid response element. Cancer Res., *56:* 483–489, 1996.

27. Joseloff, E., and Bowden, G. T. Regulation of the transcription factor AP-1 in benign and malignant mouse keratinocyte cells. Mol. Carcinog., *18:* 26–36, 1997.

28. Keeling, S. L., Gad, J. M., and Cooper, H. M. Mouse neogenin, a DCC-like molecule, has four splice variants and is expressed widely in the adult mouse and during embryogenesis. Oncogene, *15:* 691–700, 1997.

29. Norton, J. D., Deed, R. W., Craggs, G., and Sablitzky, F. Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol., *8:* 58–65, 1998.

30. Desprez, P. Y., Hara, E., Bissell, M. J., and Campisi, J. Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. Mol. Cell. Biol., *15:* 3398–3404, 1995.

31. Gautier, J., Solomon, M. J., Booher, R. N., Bazan, J. F., and Kirschner, M. W. cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. Cell, *67:* 197–211, 1991.

32. Galaktionov, K., and Beach, D. Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell, *67:* 1181–1194, 1991.

0021-972X/01/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 86, No. 3
Printed in U.S.A.

Diagnostic Value of the Acid-Labile Subunit in Acromegaly: Evaluation in Comparison with Insulin-Like Growth Factor (IGF) I, and IGF-Binding Protein-1, -2, and -3*

M. AROSIO, S. GARRONE, P. BRUZZI, G. FAGLIA, F. MINUTO, AND A. BARRECA

Institute of Endocrine Sciences, University of Milan, Ospedale Maggiore Istituto Ricovero e Cura a Carattere Scientifico (M.A., G.F.), I-20122 Milan; and Department of Endocrinology and Metabolism, University of Genova (S.G., A.C., F.M., A.B.), and Unit of Clinical Epidemiology and Trials, National Institute for Cancer Research (P.B.), I-16132 Genova, Italy

ABSTRACT

In normal subjects the main form of circulating insulin-like growth factor (IGF) is the 150-kDa complex. This complex is formed by the IGF peptide, the acid-stable IGF-binding protein-3 (IGFBP-3), and the acid-labile subunit (ALS). Experimental and clinical data have demonstrated that ALS is primarily under the control of GH and plays a critical role in maintaining constant levels of circulating IGF-I. In this study we evaluated ALS, IGF-I, and IGFBP-1, -2, and -3 in 45 acromegalic patients in basal conditions and, in 37 of these, twice after surgical therapy compared with 100 age- and sex-matched control subjects to estimate their value as parameter of GH secretory state.

The results demonstrated that in acromegaly before treatment all parameters (ALS, 523 ± 26; IGF-I, 129 ± 6; IGFBP-1, 0.7 ± 0.1; IGFBP-3, 234 ± 21; nmol/L; mean ± SEM) but IGFBP-2 were significantly different ($P < 0.0001$) from those in healthy subjects (ALS, 281 ± 4; IGF-I, 22 ± 1; IGFBP-1, 1.6 ± 0.1; IGFBP-3, 91 ± 3). IGF-I was more sensitive (100%) than ALS (89%), and both were more predictive of disease status than IGFBP-3, in that 27% of the patients had IGFBP-3 levels within the normal range. Considering the ALS/IGFBP-3 molar ratio, almost 55% of ALS circulated in a free form in active acromegaly. Before treatment, the IGF-I/IGFBPs (−1 + −2 + −3) molar ratio, which can be regarded as free, biologically active, IGF-I, was greatly increased (0.77 ± 0.06; $P < 0.0001$) compared with that in control subjects (0.23 ± 0.01).

After surgery, all 10 patients with controlled disease showed normalization of ALS (100% sensitivity), whereas 9 of them had normal IGFBP-3; reevaluation after varying lengths of time showed all these parameters within the normal range. In the 27 patients with active disease, IGF-I and ALS were more predictive of disease status (91% and 83% negative predictive values, respectively) than IGFBP-3 (53%).

The basal ALS concentration correlated only with IGFBP-3 ($r = 0.70$; $P < 0.001$). In postsurgery samples (first control) a statistically significant ($P < 0.001$) correlation was found between mean GH values as well as minimum GH after oral glucose tolerance test and ALS ($r = 0.72$ and 0.83, respectively), IGF-I ($r = 0.69$ and 0.77), IGFBP-3 ($r = 0.50$ and 0.72), and IGFBP-2 ($r = -0.36$ and -0.63). Similarly, IGF-I, IGFBP-3, and ALS were positively correlated among themselves and negatively correlated with IGFBP-2 ($P < 0.001$).

In conclusion, in the diagnosis of acromegaly, the measurement of total IGF-I appears to be the most sensitive parameter among the subunits of the 150K complex, and IGFBP-3 the least sensitive. For ALS, this subunit is quite sensitive and appears to be a useful parameter in reassessment after surgical treatment. (*J Clin Endocrinol Metab* **86:** 1091–1098, 2001)

ACROMEGALY IS characterized by chronic hypersecretion of GH. As GH secretion appears pulsatile in acromegalic patients, markers that reflect integrated GH secretion have been sought. The measurement of insulin-like growth factor I (IGF-I) is widely used in acromegaly for both diagnosis and follow-up (1, 2), as its circulating levels are quite stable, its synthesis shows GH dependency, and it mediates many of the effects of GH. In circulation, most IGF-I is bound to a GH-dependent complex of 150 kDa (150K complex) constituted by the IGF-I peptide, a specific IGF-binding protein (IGFBP-3), and an acid-labile subunit (ALS), which does not by itself bind IGF-I, but is necessary to reconstitute the whole complex from purified components (3–6). The ALS has been shown to be a glycosylated protein of 84–86 kDa and appears to have the role of increasing the molecular weight of the IGF-IGFBP-3 complex, which results in a prolongation of the IGF half-life to about 15 h (4, 5). This latter aspect is of great importance, as the IGF peptide does not readily cross the capillary-endothelial barrier in its 150-kDa form, and the ALS is therefore essential for ensuring constant plasma IGF levels (7). However, IGF-I measurement presents methodological problems, mostly due to its association with the IGFBPs in circulation. Current methods of minimizing IGFBP interference in routine assays are complex, troublesome, and often inefficient (8–10). The IGFBP-3 assay is simpler. However, IGFBP-3 concentrations are variable in acromegalic disease and do not seem always to reflect the activity of the disease (11, 12).

Despite the recognized GH dependence of the ALS and the stability of its circulating concentration, few data are cur-

Received April 3, 2000. Revision received August 15, 2000. Rerevision received October 12, 2000. Accepted November 22, 2000.

Address all correspondence and requests for reprints to: Dr. A. Barreca, Department of Endocrinology and Metabolism, University of Genova, Viale Benedetto XV, no 6. I-16132 Genova, Italy. E-mail: barreca@unige.it.

* This work was supported by Research Grants 9906153187–00 from Ministero dell'Università e della Ricerca Scientifica (MURST, Rome, Italy) and from Ospedale Maggiore IRCCS (Milan, Italy).

rently available with regard to its reliability as a parameter of GH secretory status, and these are often conflicting (13–15). Experimental and clinical data demonstrate that the ALS, like IGF-I, is primarily under the control of GH (16–18). Except in critically ill patients (19–21), the ALS is relatively independent of mechanisms known to influence IGF-I and IGFBP-3 secretion (22), thus suggesting that it might be a more specific parameter of GH secretory status. Moreover, as its measurement is unaffected by IGFBPs, which may interfere in many conventional IGF-I assays (23), it could offer several advantages over IGF-I in monitoring the activity of acromegalic disease. However, ALS measurement is relatively recent, and data on ALS concentrations in acromegaly are very scarce (23–25).

The aim of the present study is to compare ALS levels in a group of healthy subjects with those of a group of patients with active acromegaly. The effects of both successful and unsuccessful pituitary surgery on ALS levels, as judged by GH suppression during an oral glucose tolerance test (OGTT), are also reported. The reliability of the ALS in acromegaly will be considered in comparison with other components of the GH-IGF-I axis: GH, IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3.

Materials and Methods

Patient population and protocol

Forty-five acromegalic patients (23 women and 22 men; age, 21–64 yr) who had undergone adenomectomy were included in this study. Acromegaly was diagnosed on the basis of clinical features, high serum IGF-I concentrations compared with the age-adjusted normal range, and elevated GH levels that were not suppressible to less than 1 μg/L during an OGTT (26). The duration of the acromegalic disease ranged from 1–20 yr (median, 6 yr). All patients were off medical therapy for acromegaly, two were receiving replacement therapy with T_4, and none was taking glucocorticoids. Most patients had been newly diagnosed. Four patients who had received octreotide therapy (300 μg/day, sc) were assessed at least 1 month after therapy discontinuation. GH concentrations (mean of at least four fasting samples) were 23.6 ± 3.2 μg/L (±SEM), ranging from 2.6–78. 5 μg/L. In 37 patients PRL levels were within the normal range (<20 μg/L in women and <15 μg/L in men), whereas 8 patients had elevated PRL levels ranging from 23–108 μg/L. Gonadal function was normal in 15 men and 16 women. Six women were in the menopause and were not receiving hormone replacement therapy; 1 was amenorrheic. Seven men had reduced testosterone levels ranging from 2.4–9.1 nmol/L (normal range, 13–42). Two patients had diabetes mellitus: 1 was treated with insulin, and 1 with oral antidiabetic agents. In all patients the presence of a GH-secreting tumor was confirmed by immunohistochemical studies of surgically removed tissue.

Thirty-seven patients were reevaluated 1–6 months after surgery (first control). None of them had undergone medical therapy. Ten patients were considered controlled on the basis of GH suppression to less than 1 μg/L after OGTT and normal IGF-I concentrations compared with the age-adjusted normal range. In these 10 patients, the abnormal GH responses present before surgery (to TRH in 7, to sulpiride injected during dopamine infusion in 2, and to GnRH in 1) had also disappeared. One patient presenting with IGF-I level above the 97th percentile of the control group and nadir GH after OGTT below 1 μg/L was considered to have persistent disease. Two other patients, in whom GH decreased to 1 μg/L and not less than 1 after OGTT while abnormal GH responses were maintained, were not considered cured (27). Gonadal function normalized in 3 men and worsened in 2; all other anterior pituitary functions were unchanged. Antidiabetic therapies were unmodified.

In 4 of 10 patients with controlled disease and in 21 of 27 with active disease, further evaluation (second control) was performed 12–36 months after surgery. The uncontrolled group of patients was assessed while off medical therapy. Seven of these patients had undergone radiotherapy in the meantime.

One hundred age- and sex-matched healthy blood donors volunteered as controls (Table 1).

In all patients and normal controls, venous blood samples were taken in the morning (after overnight fasting) for the determination of IGF-I, ALS, IGFBP-1, IGFBP-2, and IGFBP-3. Serum was kept at −20 C until assayed. All pre- and postoperative samples from the same subject were assessed in the same assay.

Analytical methods

Serum GH levels were determined by an immunofluorimetric assay (AutoDelfia hGH, Wallac, Inc., Turku, Finland). The sensitivity of the assay was 0.01 μg/L; the intra- and interassay coefficients of variation were 2.6% and 3.9%, respectively.

IGF-I was measured by RIA using immunochemicals and tracer provided by Medgenix (Fleurus, Belgium). The sensitivity of the assay was 1.2 nmol/L; the intra- and interassay coefficients of variation were 6% and 7.5%, respectively. To avoid interference from binding proteins, single plasma ethylenediamine tetraacetate samples were treated with acid-ethanol according to the method of Daughaday *et al.* (28).

Serum total ALS was measured by means of specific two-site sandwich enzyme-linked immunosorbent assay, using anti-ALS antibodies raised against synthetic amino-terminal and carboxyl-terminal ALS peptides, and reagents and tracer provided by Diagnostics Systems Laboratories, Inc. (Webster, TX). All samples were pretreated to dissociate the complexed ALS and enhance ALS immunoreactivity. The sensitivity of the assay was 4.7 nmol/L; the intra- and interassay coefficients of variation were 5.5% and 7.2%, respectively. Recovery of human serum-derived glycosylated ALS [purified as described previously (6)] was 75% for the lower concentration added (1 μg) and 95% for the higher concentration added (60 μg).

IGFBP-1 was measured by immunoradiometric assay, using reagents and tracer provided by Diagnostics Systems Laboratories, Inc. The sensitivity of the assay was 0.05 nmol/L; the intra- and interassay coefficients of variation were 2.5% and 4.6%, respectively.

IGFBP-2 levels were determined by double antibody RIA using a nonequilibrium technique, as described by Clemmons *et al.* (29). Specific IGFBP-2 antiserum was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and the standard was a pure IGFBP-2 preparation obtained by DNA recombinant technology (ImmunoKontact, Frankfurt, Germany). The sensitivity of the assay was 0.01 nmol/L; the intra- and interassay coefficients of variation were 6% and 9.5%, respectively.

IGFBP-3 was measured by immunoassay, using reagents and tracer

TABLE 1. Comparison between hormone levels in healthy subjects and in untreated acromegalic patients

Parameters	Healthy subjects	Acromegalic patients	*P*
No. of cases	100	45	
Sex (F/M)	0/50	23/22	
Age (yr)	43 ± 12 (40–45)	39 ± 11 (36–42)	NS
ALS (nmol/L)	281 ± 43 (273–290)	523 ± 171 (473–573)	<0.0001
IGF-I (nmol/L)	22 ± 4 (21–23)	129 ± 39 (118–140)	<0.0001
IGFBP-1 (nmol/L)	1.6 ± 1.2 (1.4–1.9)	0.71 ± 0.6 (0.5–0.9)	<0.0001
IGFBP-2 (nmol/L)	8.6 ± 5.4 (7.6–9.7)	7.5 ± 5.8 (5.7–9.1)	NS
IGFBP-3 (nmol/L)	91 ± 29 (86–97)	234 ± 141 (193–275)	<0.0001

Data are shown as the mean ± SD; 95% confidence intervals are given in *parentheses*.

provided by Diagnostics Systems Laboratories, Inc. All samples were diluted appropriately so as to reach a point in the curve where there is parallelism among unglycosylated *Escherichia coli*-derived IGFBP-3, glycosylated Chinese hamster ovary-derived IGFBP-3, and serum (%B/B0, 70–85%). The sensitivity of the assay was 0.04 nmol/L; the intra- and interassay coefficients of variation were 3.25% and 5.6%, respectively.

Statistical analysis

Statistical analysis was performed by nonparametric test on paired (Wilcoxon signed rank test) and unpaired (Mann-Whitney test) observations. $P < 0.05$ was considered significant. Results are expressed as the mean ± SEM. The correlations among all parameters studied were evaluated using Spearman rank order statistics and linear regression analysis models. For this purpose, log-transformed values of GH and IGFBP-3, which showed a log normal distribution, were used. The 97th percentile of the normal distribution calculated for each age group in the healthy subjects was chosen as the cut-off point for normal serum levels of all parameters of the IGF system. Because normal levels are largely arbitrary and vary according to laboratory, we investigated the diagnostic accuracy of each parameter at different cut-off points by determination of the receiver-operating characteristic (ROC). All statistical analyses were made using SPSS statistical software (SPSS, Inc., Chicago, IL).

Results

Healthy subjects

The mean concentrations of the different parameters are shown in Table 1 and Fig. 1. Analytical distribution of age and single values of IGF-I, IGFBP-3, and ALS are shown in Fig. 2.

As expected, a negative correlation between IGF-I and age and between IGFBP-3 and age ($r = -0.61$ for IGF-I and $r = -0.40$ for IGFBP-3; $P < 0.001$ for both) was observed. No statistically significant correlation was found between ALS levels and age in the 20–65 yr age range. Indeed, a statistically significant difference ($P < 0.001$) between 20- to 35-yr-old subjects and 50- to 65-yr-old subjects was present for IGF-I, IGFBP-2, and IGFBP-3, but not for ALS. IGFBP-2 levels significantly increased with age in women, but not in men ($r = 0.51$; $P < 0.01$). No other gender differences were found. ALS concentrations were significantly correlated with IGF-I ($P < 0.0001$) and IGFBP-3 levels ($P < 0.001$). The ALS/IGFBP-3 molar ratio was 3.4 ± 0.1. Overall, the 3rd and 97th percentiles were 13.3 and 30.1 nmol/L for IGF-I, 216 and 350 nmol/L for ALS, 50 and 136 nmol/L for IGFBP-3, 0.2 and 3.9 nmol/L for IGFBP-1, and 2.4 and 18.7 nmol/L for IGFBP-2.

Acromegalic patients: before surgery

In the group of acromegalic patients ALS levels were elevated before transsphenoidal surgery (Table 1 and Figs. 1 and 2). ALS levels were higher than the 97th percentile of healthy subjects in 40 patients (89%). One of the 5 patients in

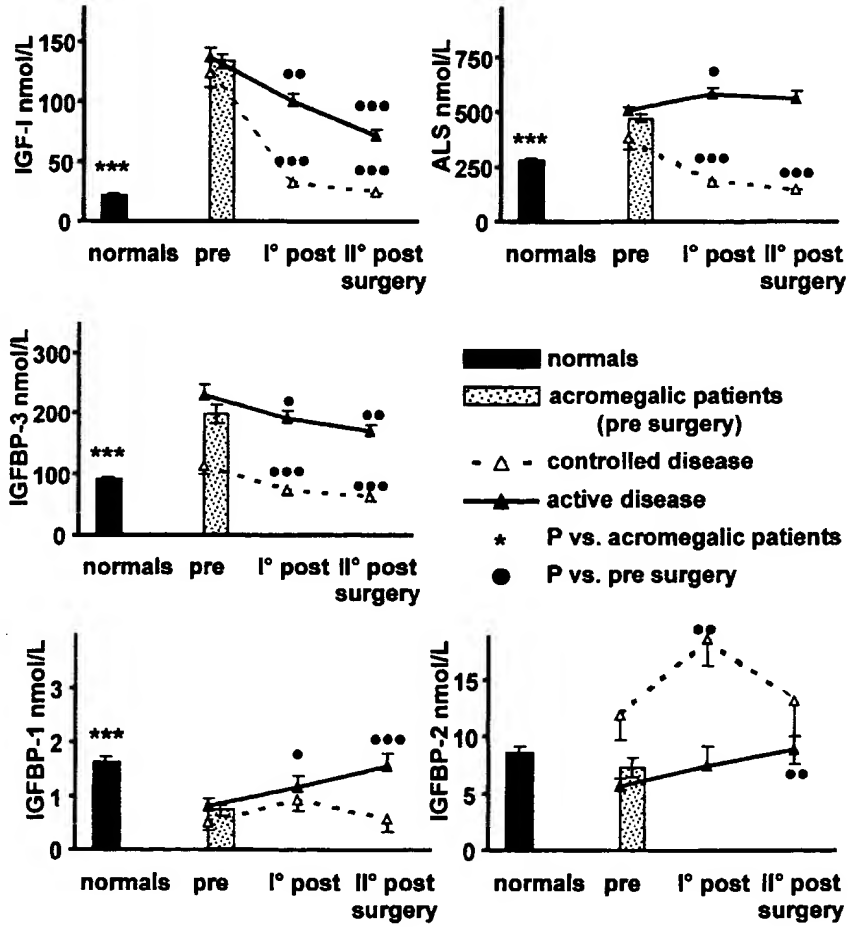

FIG. 1. Serum levels of IGF-I, ALS, IGFBP-3, IGFBP-1, and IGFBP-2 in 100 normal subjects, 45 acromegalic patients before surgery, and 37 acromegalic patients after surgery (11 with controlled and 26 with active disease). Data are shown as the mean ± SEM. ●, $P < 0.05$; ●●, $P < 0.001$; ●●● or ***, $P < 0.0001$.

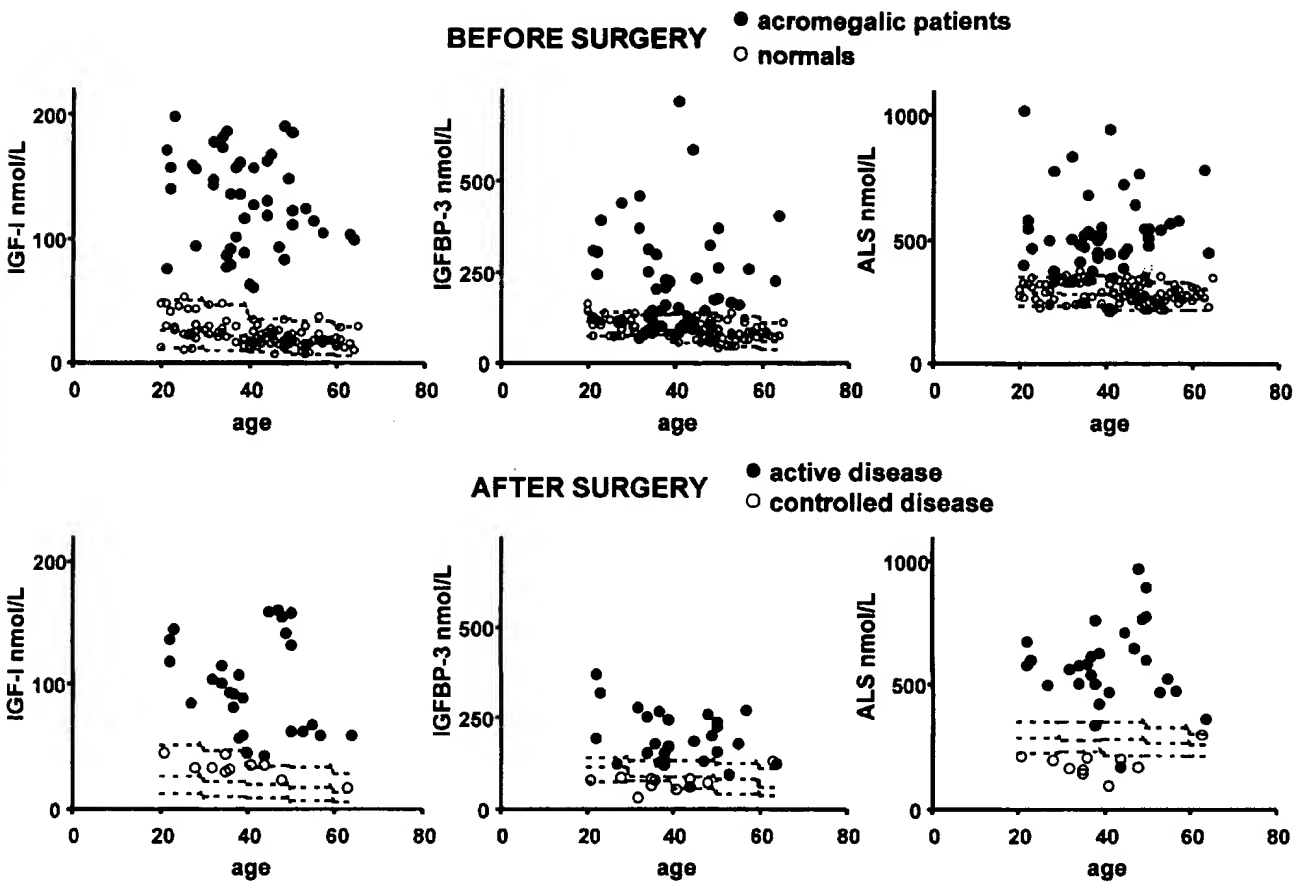

FIG. 2. Serum IGF-I, IGFBP-3, and ALS in normal subjects and in 45 acromegalic patients before and after surgery. *Dashed lines* represent the 3rd, 50th, and 97th percentiles of the control group.

whom ALS levels were below the 97th percentile of healthy controls also showed the lowest concentration of GH (mean value, 2.6 μg/L) and response after OGTT (1.4 μg/L). IGF-I concentration, although always above the normal range for age, tended to be lower in these patients than in patients with high ALS levels. All 5 also showed IGFBP-3 below the 97th percentile and IGFBP-2 levels in the higher range concentration. The basal ALS concentration correlated with IGFBP-3 ($r = 0.70$; $P < 0.001$). No correlation with age, GH, IGF-I, IGFBP-1, IGFBP-2, or tumor size was observed in untreated acromegalic patients. No differences according to gender, PRL levels, or gonadal status were observed.

IGF-I levels were elevated in all patients compared with those in normal controls (Table 1 and Figs. 1 and 2), ranging from 60–197 nmol/L. The IGF-I/IGFBPs ($-1 + -2 + -3$) molar ratio was significantly ($P < 0.001$) higher (0.77 ± 0.1) than that in normal subjects (0.23 ± 0.01). In contrast with healthy subjects, the IGF-I concentration did not decrease with age. A significant correlation was found between IGF-I and GH mean concentrations and nadir value after OGTT ($r = 0.44$ and 0.59; $P < 0.01$).

IGFBP-3 levels ranged from 67–717 nmol/L (Table 1 and Figs. 1 and 2). About two thirds of the patients showed high IGFBP-3 concentrations, whereas 12 (27%) had IGFBP-3 levels in the normal range. No correlations were found with the parameters evaluated, apart from ALS.

IGFBP-1 concentrations ranged from 0.08–2.46 nmol/L and were significantly lower than IGFBP-1 levels in normal controls (Table 1 and Fig. 1).

IGFBP-2 levels ranged from 1.44–26.37 nmol/L and were not statistically different from those in controls (Fig. 1). However, with regard to the IGFBP-2/IGF-I molar ratio, 95% of patients showed a value below the third percentile of that in healthy subjects.

None of the parameters considered, apart from GH concentration ($P < 0.01$), correlated with the size of the adenoma.

Information on the discriminatory ability of each parameter evaluated by ROC curve analysis (Fig. 3) was in close agreement with the estimates obtained using the cut-off points from normal controls (97th percentile).

No ROC curve for IGF-I could be obtained, as discrimination between acromegalic patients and normal controls was total, with no overlapping of the values in the two groups. The areas under the curve for ALS, IGFBP-3, IGFBP-1, and IGFBP-2 were 0.937, 0.892, 0.798, and 0.584, respectively, indicating that the parameter with the best discrimination, after IGF-I, was ALS, followed by IGFBP-3.

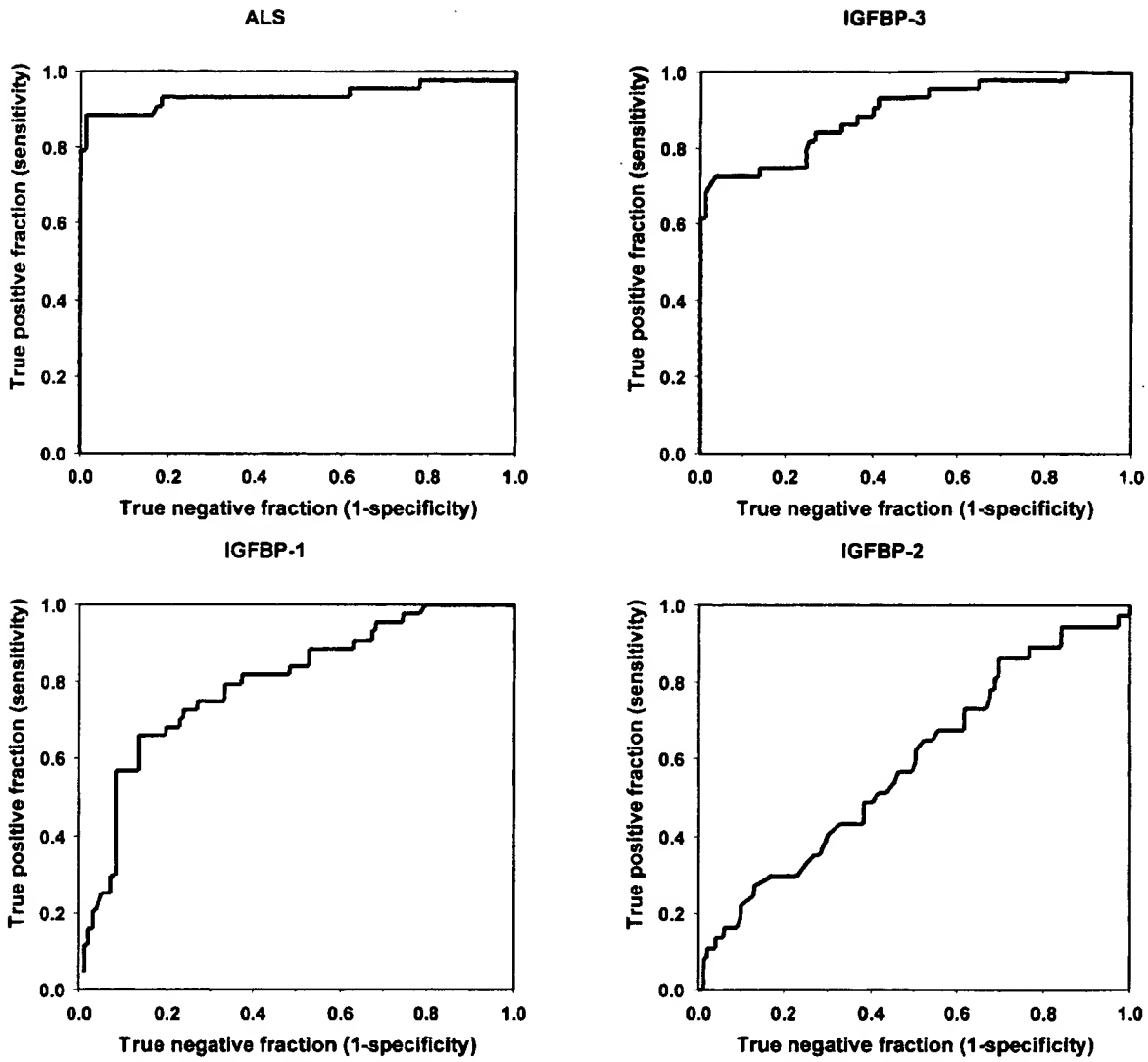

FIG. 3. ROC assessed the comparative diagnostic performance of ALS, IGFBP-3, IGFBP-1, and IGFBP-2. The ROC curve for IGF-I cannot be estimated because there was no overlap between values in cases and in controls (sensitivity, 100%; specificity, 100%).

Acromegalic patients: reevaluation after surgery

Thirty-seven patients were reevaluated after transsphenoidal surgery. Mean levels of the different parameters before and after surgery are shown in Fig. 1. All parameters showed the expected modifications after removal or reduction of the adenoma; GH, IGF-I, and IGFBP-3 decreased, whereas IGFBP-1 and IGFBP-2 increased. The sensitivity, specificity, and positive and negative predictive values of ALS, IGF-I, and IGFBP-3 in identifying patients with controlled and active disease are shown in Table 2.

Surgery was considered successful in 10 patients, in whom an oral glucose tolerance test suppressed GH concentrations below 1 μg/L, and IGF-I was normalized. Before surgery, this group of patients had significantly lower levels of ALS (381 $\pm$ 52 *vs.* 510 $\pm$ 15; $P < 0.002$) and IGFBP-3 (112 $\pm$ 14 *vs.* 230 $\pm$ 17; $P < 0.0001$) and significantly higher levels of IGFBP-2 (12.0 $\pm$ 2.1 *vs.* 5.7 $\pm$ 0.6; $P < 0.005$) than patients in whom surgery was unsuccessful. After successful surgery, 10 controlled patients showed normal concentrations of ALS, thus confirming good control of the disease, whereas one patient presented with an IGFBP-3 concentration slightly above the 97th percentile of the control group (Fig. 2 and Table 2). In 4 controlled patients, reevaluation after varying lengths of time (control 2) showed IGF-I, IGFBP-3, and ALS concentrations in the normal range.

Twenty-six of the 27 patients in whom surgery was unsuccessful had elevated IGF-I concentrations, whereas 2 (7%) and 8 (30%) showed normal ALS and IGFBP-3, respectively (Fig. 2 and Table 2). Reevaluation of the only patient who had presented with IGF-I, IGFBP-3, and ALS concentrations at the upper end of the normal range at the first control revealed increased concentrations of IGF-I and ALS, but not IGFBP-3. The other patient with ALS below the normal range, but IGF-I above the 97th percentile of the control group, showed

TABLE 2. Sensitivity, specificity, and predictive values of IGF-I, IGFBP-3, and ALS in 37 acromegalic patients reevaluated 1–6 months after surgery

	All patients (n = 37)	Patients with controlled disease (n = 10)		Patients with active disease (n = 27)		PPV	NPV
IGF-I		Specificity, 100%	False positive, 0%	Sensitivity, 96%	False negative, 4%		
Normal	11 (30%)	n = 10			n = 1		91%
Elevated	26 (70%)		n = 0	n = 26		100%	
IGFBP-3		Specificity, 90%	False positive, 10%	Sensitivity, 70%	False negative, 30%		
Normal	17 (46%)	n = 9			n = 8		53%
Elevated	20 (54%)		n = 1	n = 19		95%	
ALS		Specificity, 100%	False positive, 0%	Sensitivity, 93%	False negative, 7%		
Normal	12 (32%)	n = 10			n = 2		83%
Elevated	25 (68%)		n = 0	n = 25		100%	

Data are given as the percentage of outcome of treatment. PPV and NPV, Positive and negative predictive values, respectively.

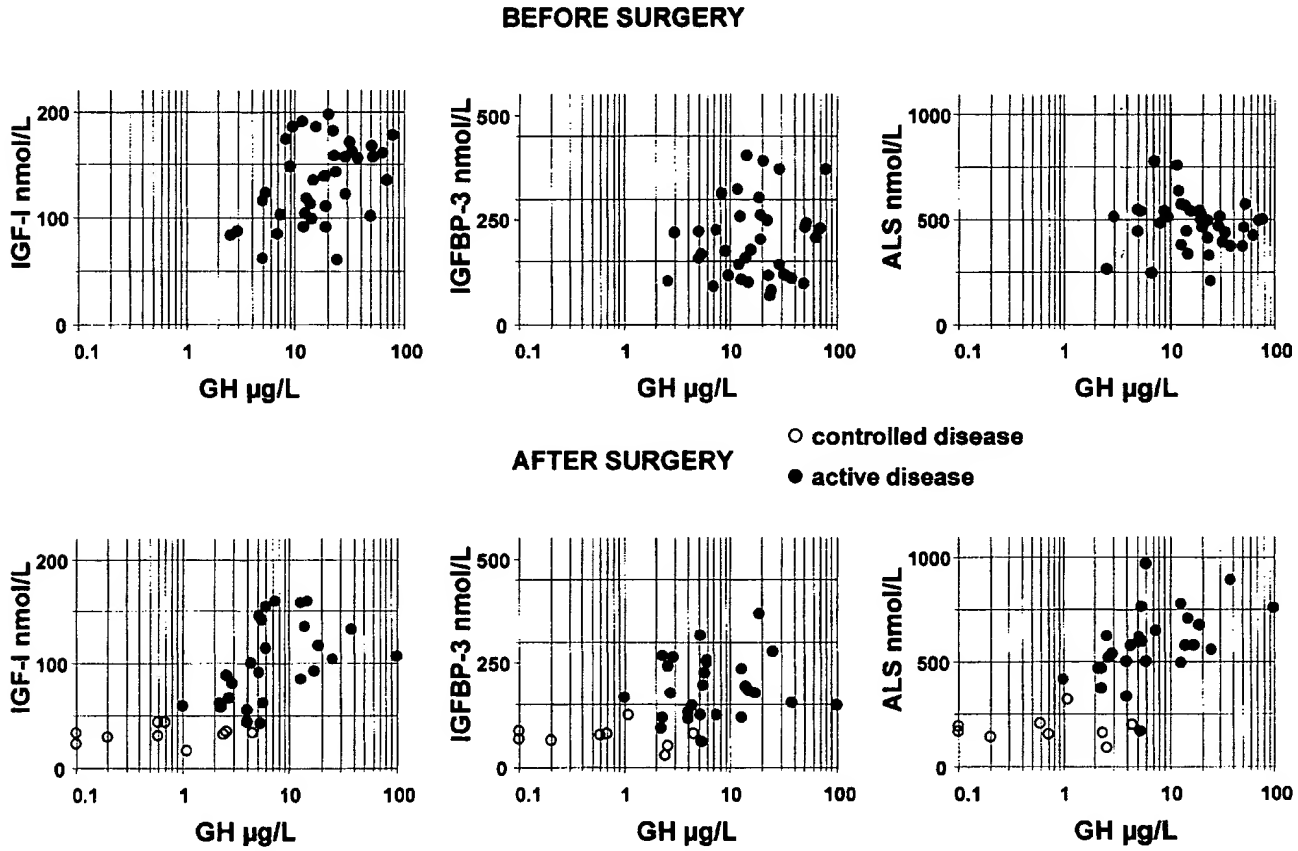

FIG. 4. Correlations between IGF-I, ALS, and IGFBP-3 concentrations and mean GH concentration in acromegalic patients before and after transsphenoidal surgery.

a nadir GH less than 1 µg/L after an OGTT and normal IGFBP-3. Reevaluation of this patient showed normalization of all parameters, including IGF-I concentration. Therefore, in 20 of 21 patients who were reevaluated (control 2), elevated concentrations of IGF-I and ALS persisted, ranging from 60–120 and from 382–976 nmol/L, respectively, whereas IGFBP-3 levels were normal in 5 patients.

IGFBP-1 levels increased slightly, although not significantly, in both controlled and uncontrolled patients (Fig. 1).

IGFBP-2 levels and IGFBP-2/IGF-I ratio increased significantly only in the group of controlled patients (Table 1 and Fig. 1). All patients with controlled disease showed a significantly increased ($P < 0.001$) IGFBP-2/IGF-I ratio in both postsurgical follow-up examinations, whereas 23 of 26 patients with active disease showed a ratio lower than the third percentile of that of the normal population.

In postsurgery samples (first control) a statistically significant ($P < 0.001$) correlation was found between mean GH values (Fig. 4) as well as minimum GH after OGTT and ALS ($r = 0.72$ and 0.83, respectively), IGF-I ($r = 0.69$ and 0.77), IGFBP-3 ($r = 0.46$ and 0.67), and IGFBP-2 ($r = -0.36$ and -0.63). Similarly, IGF-I, IGFBP-3, and ALS were positively correlated among themselves and negatively correlated with IGFBP-2 ($P < 0.001$).

Discussion

As the subunits of the 150K complex are GH dependent and stable in circulation, they constitute a sensitive and specific index of integrated 24-h GH secretion, and their use can avoid causing the patient the stress of multiple sampling for evaluation of the GH secretory state. Several studies have established the high specificity and sensitivity of the total IGF-I concentration in the diagnosis of acromegaly (1, 2, 30), whereas data on the reliability of IGFBP-3 are conflicting, and those on ALS are scant, even though their measurement is less difficult, as it does not require previous extraction. Unfortunately, some well known limitations of IGF-I, resulting from its age dependency and its modification in some clinical conditions (malnutrition, diabetes, hypothyroidism, and chronic renal failure) are more or less shared by the other subunits of the 150K complex. All of these limitations, however, can easily be overcome by means of specific testing for individual diseases and by defining normal ranges with reference to a large number of age- and sex-matched control subjects. In this respect it is noteworthy that ALS levels in 20- to 65-yr-old normal subjects appear to be less age dependent than IGF-I and IGFBP-3. This finding is in agreement with the data reported by Juul *et al.* (31) in adult women, although we did not find a significant decrease in ALS levels in adult men. Our data in normal subjects confirm that the molar concentration of ALS in the circulation exceeds that of IGFBP-3 by about two thirds (23), and that its concentration is positively correlated with IGF-I and IGFBP-3 concentrations (25).

The present study, carried out by comparing a large number of acromegalic patients with 100 healthy adults, shows that active acromegaly is characterized by elevated ALS concentrations. Previous data are concordant with our finding that ALS levels in acromegalic patients are about twice those present in normal subjects. Indeed, using an antibody raised against serum-derived glycosylated ALS, Baxter (24) first showed that ALS levels were elevated in acromegalic patients and, using the same RIA, Hoffman *et al.* (15) reported mean ALS levels 2.5-fold above the normal mean and 91% of patients with values above the normal range. More recently, using the same two-site sandwich enzyme-linked immunosorbent assay as we used, Khosravi *et al.* (25) found mean ALS levels in 20 acromegalics about 1.6-fold above the mean of control value, and 80% of patients above the normal range. In the 45 acromegalic patients evaluated in this study, pathological ALS levels were found in 89% of patients before treatment. In the remaining 5 patients, we could not find any known cause shared by all to justify ALS levels within the normal range. However, we cannot exclude that the complex regulatory mechanisms modulating the circulating levels of the 150K complex (16–22) might differently affect the synthesis of each subunit, at least in some patients. Nevertheless, in this subset of patients GH levels and IGF-I concentrations tended to be lower, and IGFBP-2 levels higher, than in patients with pathological ALS levels. The finding that all of them also showed IGFBP-3 below the normal range confirms the close association of these two components of the 150K complex. Indeed, the findings that multiple linear regression analysis indicates that ALS is the factor most closely associated with IGFBP-3 concentration ($P < 0.005$) and that ALS/IGFBP-3 molar ratio found in acromegalic patients does not differ significantly from that seen in normal subjects are in agreement with the hypothesis that much of the apparent GH dependency of serum IGFBP-3 might be secondary to its stabilization by ALS (32). Considering the ALS/IGFBP-3 molar ratio, almost 55% of ALS should circulate in a free form in acromegaly. In agreement with data reported by Juul *et al.* (33), not only the IGF-I/IGFBP-3 molar ratio but also the IGF-I/IGFBPs ($-1 + -2 + -3$) ratio, which can be regarded as free, biologically active, IGF-I, was greatly increased in patients with active acromegaly. This increase was more closely related to the increased IGF-I synthesis and the elevated IGF-I/IGFBP-3 molar ratio than to the reduction of the IGFBPs negatively regulated by GH. Indeed, IGFBP-2 was unchanged, and IGFBP-1 was, on the average, only half that seen in control subjects.

In the acromegalic patients before treatment, the levels of total IGF-I and IGFBP-3 were increased in 100% and 73% of patients, respectively. The finding regarding the poor reliability of IGFBP-3 in the diagnosis of acromegaly is in agreement with several previous reports showing that from 26–45% of patients have IGFBP-3 levels overlapping with those of healthy controls (11, 12, 34, 35), although in one study (36) elevated levels of IGFBP-3 were found in all 18 untreated acromegalic patients.

In postsurgical reassessment of the disease, it is noteworthy that ALS shows a positive predictive value equal to that of IGF-I (100%) and a negative predictive value slightly lower (83%) than that of IGF-I (91%), whereas IGFBP-3 again shows the lowest sensitivity and specificity.

Although GH exerts an inhibitory effect on the synthesis of IGFBP-1 as well as IGFBP-2 (29, 38), IGFBP-1 is not usually considered, because, unlike IGFBP-3 and IGFBP-2, it shows marked diurnal variations due to changes in metabolic status (38). Moreover, owing to its short half-life and lower circulating levels, IGFBP-1 does not usually play an important role in stabilizing circulating IGFs (39). The observation that IGFBP-1 levels were lower in acromegaly than in normal subjects is in agreement with previous reports (33, 40, 41) and with the known inhibition of this parameter by insulin (38). After surgery, IGFBP-1 levels increased, reaching statistical significance only in the group of controlled patients. Jørgensen *et al.* (41) found serum IGFBP-1 levels to be slightly supernormal after adenomectomy; this finding was not confirmed by our data, according to which IGFBP-1 only normalized.

Although IGFBP-2 levels significantly lower than those in healthy subjects have been reported in acromegalics (33, 41, 42), our data, in agreement with those reported by Clemmons *et al.* (29), showed IGFBP-2 concentrations not significantly different from those found in a large group of normal subjects. With regard to the IGFBP-2/IGF-I molar ratio, 94% of patients showed a ratio below the third percentile of healthy subjects, and this ratio normalized in controlled patients. After surgery, IGFBP-2 greatly increased in the group of controlled patients, without any significant change in not controlled patients, thus suggesting that elevated GH and IGF-I indeed have an effect on IGFBP-2.

In postoperative samples, GH secretion (mean values as well as nadir GH after OGTT) correlated mostly with ALS and IGF-I and to a lesser extent with IGFBP-3. Our previous finding of a log-linear correlation between GH and IGF-I (2) is further corroborated by this study and extended to the ALS subunit and to IGFBP-3.

In conclusion, our data show that active acromegaly is characterized by elevated ALS concentrations as well as elevated total and free IGF-I levels. Mean levels of IGFBP-3 were also higher, whereas levels of IGFBP-1 were lower than those in normal subjects. However, one quarter of the patients had IGFBP-3 levels in the normal range, and most of these showed normal IGFBP-1 and IGFBP-2 concentrations. These observations are equally true when considering newly diagnosed patients and patients with active disease after pituitary surgery. Therefore, in the diagnosis of acromegaly, the measurement of total IGF-I appears to be the most sensitive parameter among the subunits of the 150K complex, and IGFBP-3 the least sensitive. For what concerns ALS, this subunit is seen to be quite sensitive and appears to be a useful parameter in reassessment after surgical treatment.

References

1. **Clemmons DR, VanWyk JJ, Ridgway EC, Kliman B, Kjellberg RN, Underwood LE.** 1979 Evalation of acromegaly by radioimmunoassay of somtomedin-C. N Engl J Med. 301:1138–1142.
2. **Barreca A, Ciccarelli E, Minuto F, Bruzzi P, Giordano G, Camanni F.** 1989 Insulin-like growth factor I and daily growth hormone profile in the assessment of active acromegaly. Acta Endocrinol (Copenh). 120:629–635.
3. **Furlanetto RW.** 1980 The somatomedin C binding protein: evidence for a heterologous subunit structure. J Clin Endocrinol Metab. 51:12–19.
4. **Baxter RC.** 1988 Characterization of the acid-labile subunit of the growth hormone-dependent insulin-like growth factor binding protein complex. J Clin Endocrinol Metab. 67:265–272.
5. **Baxter RC, Martin JL, Beniac VA.** 1989 High molecular weight insulin-like growth factor binding protein complex. Purification and properties of the acid-labile subunit from human serum. J Biol Chem. 264:11843–11848.
6. **Barreca A, Ponzani P, Arvigo M, Giordano G, Minuto F.** 1995 Effect of the acid-labile subunit on the binding of insulin-like growth factor (IGF)-binding protein-3 to [125I]IGF-I. J Clin Endocrinol Metab. 80:1318–1324.
7. **Zapf J, Schmid CH, Froesch ER.** 1984 Biological and immunological properties of insulin-like growth factors (IGF) I and II. Clin Endocrinol Metab. 13:3–30.
8. **Daughaday WH, Kapadia M, Mariz IK.** 1986 Serum somatomedin binding proteins: physiologic significance and interference in radioligand assay. J Lab Clin Med 109:335–363.
9. **Mohan S, Baylink D.** 1995 Development of a simple valid method for the complete removal of insulin-like growth factor (IGF)-binding proteins from IGFs in human serum and other biological fluids: comparison with acid-ethanol treatment and C_{18} Sep-Pak separation. J Clin Endocrinolo Metab. 80:637–647.
10. **Rosenfeld RG, Gargosky SH.** 1996 Assays of insulin-like growth factors and their binding proteins:practicalities and pitfalls. J Pediatr. 128:S52–S57.
11. **De Herder WW, Van der Lely AJ, Janssen JAMJL, Uitterlinden P, Hofland LJ, Lamberts SWJ.** 1995 IGFBP-3 is a poor parameter for assessment of clinical activity in acromegaly. Clin Endocrinol (Oxf). 43:501–505.
12. **Thissen JP, Ketelslegers JM, Maiter D.** 1996 Use of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 in the diagnosis of acromegaly and growth hormone deficiency in adults. Growth Regul. 6:222–229.
13. **de Boer H, Blok GJ, Popp-Snijders C, Stuurman L, Baxter RC, van der Veen E.** 1996 Monitoring of growth hormone replacement therapy in adults, based on measurement of serum markers. J Clin Endocrinol Metab. 81:1371–1377.
14. **Thoren M, Hilding A, Baxter RC, Degerblad M, Wivall-Helleryd IL, Hall K.** 1997 Serum insulin-like growth factor I (IGF-I), IGF-binding protein-1 and -3, and the acid-labile subunit as serum markers of body composition during growth hormone (GH) therapy in adults with GH deficiency. J Clin Endocrinol Metab. 82:223–228.
15. **Hoffman DM, Baxter RC, O'Sullivan AJ, Crampton L, Ho K.** 1997 Serum acid-labile subunit in adult growth hormone deficiency and acromegaly [Abstract]. Endocrinol Metab. 4(Suppl A):23.
16. **Chin E, Zhou J, Dai J, Baxter RC, Bondy CA.** 1994 Cellular localization and regulation of gene expression for components of the insulin-like growth factor ternary binding protein complex. Endocrinology. 134:2498–2504.
17. **Dai J, Baxter RC.** 1994 Regulation *in vivo* of the acid-labile subunit of the rat serum insulin-like growth factor-binding protein complex. Endocrinology. 135:2335–2341.
18. **Ooi GT, Cohen FJ, Tseng LYH, Rechler MM, Boisclir YR.** 1997 Growth hormone stimulates transcription of the gene encoding the acid-labile subunit (ALS) of the circulating insulin-like growth factor-binding protein complex and ALS promoter activity in rat liver. Mol Endocrinol. 11:997–1007.
19. **Bereket A, Wilson TA, Blethen SL, et al.** 1996 Regulation of the acid-labile subunit of the insulin-like growth factor ternary complex in patients with insulin-dependent diabetes mellitus and severe burns. Clin Endocrinol (Oxf). 44:525–532.
20. **Barreca A, Ketelslegers JM, Arvigo M, Minuto F, Thissen JP.** 1998 Decreased acid-labile subunit (ALS) levels by endotoxin *in vivo* and by interleukin-1β *in vitro*. Growth Horm IGF Res. 8:217–223.
21. **Baxter RC, Hawker FH, To C, Stewart PM, Holman SR.** 1997 Thirty-day monitoring of insulin-like growth factors and their binding proteins in intensive care unit patients. Growth Reg. 7:1–11.
22. **Barreca A, Voci A, Lee PDK, et al.** 1997 Effect of the somatostatin analog, octreotide, and of other hormones on the release of the acid-labile subunit (ALS) of the 150kDa complex by rat hepatocytes in primary culture. Eur J Endocrinol. 137:193–199.
23. **Baxter RC.** 1997 Editorial: the binding protein's binding protein: clinical applications of acid-labile subunit (ALS) measurement. J Clin Endocrinol Metab. 82:3941–3943.
24. **Baxter RC.** 1990 Circulating levels and molecular distribution of the acid-labile (α) subunit of the high molecular weight insulin-like growth factor-binding protein complex. J Clin Endocrinol Metab. 70:1347–1353.
25. **Khosravi MJ, Diamandi A, Mistry J, Krishna RG, Khare A.** 1997 Acid-labile subunit of human insulin-like growth factor binding protein complex: measurement, molecular and clinical evaluation. J Clin Endocrinol Metab. 82:3944–3951.
26. **Giustina A, Barkan A, Casanueva FF, et al.** 2000 Criteria for cure of acromegly: a consensus statement. J Clin Endocrinol Metab. 85:526–529.
27. **Arosio M, Giovanelli MA, Riva E, Nava C, Ambrosi B, Faglia G.** 1983 Clinical use of pre- and postsurgical evaluation of abnormal GH responses in acromegaly. J Neurosurg. 59:402–408.
28. **Daughaday WH, Mariz IK, Blethen SL.** 1980 Inhibition of access of bound somatomedin to membrane receptor and immunoglobin sites: a comparison of radioreceptor and radioimmunoassay of somatomedin in native and acid-ethanol extracted serum. J Clin Endocrinol Metab. 51:781–788.
29. **Clemmons DR, Snyder DK, Busby Jr WH.** 1991 Variables controlling the secretion of insulin-like growth factor binding protein-2 in normal human subjects. J Clin Endocrinol Metab. 73:727–733.
30. **Lamberts SW, Uitterlinden P, Verleun T.** 1987 Relationship between growth hormone and somatomedin-C levels in untreated acromegaly, after surgery and radiotherapy and during medical therapy with sandostatin (SMS 201–995). Eur J Clin Invest. 17:354–359.
31. **Juul A, Møller S, Mosfeldt-Laursen E, et al.** 1998 The acid-labile subunit of human ternary insulin-like growth factor binding protein complex in serum: hepatosplanchnic release, diurnal variation, circulating concentrations in healthy subjects, and diagnostic use in patients with growth hormone deficiency. J Clin Endocrinol Metab. 83:4408–4415.
32. **Olivecrona H, Hilding A, Ekström C, et al.** 1999 Acute and short-term effects of growth hormone on insulin-like growth factors and their binding proteins: serum levels and hepatic messenger ribonucleic acid responses in humans. J Clin Endocrinol Metab. 84:553–560.
33. **Juul A, Main K, Blum WF, Lindholm J, Ranke MB, ND Skakkebaek NE.** 1994 The ratio between serum levels of insulin-like growth factor (IGF)-I and the IGF binding proteins (IGFBP-1, 2 and 3) decreases with age in healthy adults and increases in acromegalic patients. Clin Endocrinol (Oxf). 41:85–93.
34. **Paramo C, Andrade MAO, Fluiters E, Luna R, de la Fuente J, Garcia-Mayor RV.** 1997 Comparative study of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 (IGFBP-3) level and IGF-I/IGFBP-3 ratio measurements and their relationship with an index of clinical activity in the management of patients with acromegaly. Metabolism. 46:494–498.
35. **Stoffel-Wagner B, Springer W, Bidlingmaier F, Klingmüller D.** 1997 A comparison of different methods for diagnosing acromegaly. Clin Endocrinol (Oxf). 46:531–537.
36. **Grinspoon S, Clemmons D, Swearingen B, Klibanski A.** 1995 Serum insulin-like growth factor-binding protein-3 levels in the diagnosis of acromegaly. J Clin Endocrinol Metab. 80:927–932.
37. **Deleted in proof.**
38. **Lee PD, Giudice LC, Conover CA, Powell DR.** 1997 Insulin-like growth factor binding protein-1: recent findings and new directions. Proc Soc Exp Biol Med. 216:319–357.
39. **Zapf J.** 1995 Physiological role of the insulin-like growth factor binding proteins. Eur J Endocrinol. 132:645–654.
40. **Holly JMP, Cotterill AM, Jemmott RC, et al.** 1991 Inter-relations between growth hormone, insulin, insulin-like growth factor-I (IGF-I), IGF-binding protein-1 (IGFBP-1) and sex hormone-binding globulin in acromegaly. Clin Endocrinol (Oxf). 34:275–280.
41. **Jørgensen JOL, Møller N, Møller J, Weeke J, Blum WF.** 1994 Insulin-like growth factors (IGF)-I and -II and IGF binding protein-1, -2, and -3 in patients with acromegaly before and after adenomectomy. Metabolism. 43:579–583.
42. **Blum WF, Horn N, Kratzsch J, et al.** 1993 Clinical studies of IGFBP-2 by radioimmunoassay. Growth Regul. 3:100–104.

0013-7227/00/$03.00/0
Endocrinology

Vol. 141, No. 2
Printed in U.S.A.

Conservation of a Growth Hormone-Responsive Promoter Element in the Human and Mouse Acid-Labile Subunit Genes*

ADISAK SUWANICHKUL, YVES R. BOISCLAIR, ROBERT C. OLNEY, SUSAN K. DURHAM, AND DAVID R. POWELL

Department of Pediatrics, Baylor College of Medicine (A.S., S.K.D., D.R.P.), Houston, Texas 77030; the Department of Animal Science, Cornell University (Y.R.B.), Ithaca, New York 14853; the Department of Pediatrics, The Nemours Children's Clinic, Mayo Medical School (R.C.O.), Jacksonville, Florida 32207

ABSTRACT

During extrauterine life, insulin-like growth factors (IGFs) circulate in a ternary serum complex with one IGF-binding protein-3 (IGFBP-3) or IGFBP-5 protein and with a single acid-labile subunit (ALS). GH increases levels of this ternary complex; in mice, this effect is achieved in part by the ability of GH to stimulate mouse ALS (mALS) transcription through an interferon-γ-activated sequence-like element (GLE) in the mALS promoter. To begin studying how GH regulates human ALS (hALS) gene expression, we cloned the hALS gene and found that it spans approximately 3.3 kb of DNA at chromosomal region 16p13.3. The hALS gene has two exons separated by a 1235-bp intron, which is found at the identical site in rat and mouse ALS genes. Sequence analysis reveals that the hALS 5′-flanking sequence is homologous to the mALS promoter, and that the GH-responsive GLE in the mALS promoter is conserved in both sequence and location in the hALS gene. The region spanning from −755 to −4 bp 5′ to the hALS ATG translation start codon directs expression of a luciferase reporter gene in primary rat hepatocytes, and GH increases reporter expression in the presence of the native, but not a mutant, GLE in the hALS promoter. These data suggest that GH stimulates hALS and mALS gene expression by a similar mechanism, which involves at least in part a conserved GLE in the ALS promoter. (*Endocrinology* **141:** 833–838, 2000)

INSULIN-LIKE growth factor I (IGF-I) and IGF-II are approximately 7.5-kDa proteins that exhibit mitogenic, metabolic, differentiative, chemotactic, and antiapoptotic effects on many tissues and cell types (1, 2). IGFs often confer their effects in an autocrine/paracrine manner (1, 2). After birth, however, circulating IGFs also appear to be biologically important, as many effects of GH are mediated by IGF-I (1–3).

IGFs circulate in serum and other body fluids at higher molecular mass, tightly bound by a family of at least six IGF-binding proteins (IGFBPs) (4–6). During extrauterine life, most IGFs circulate at about 150 kDa in a ternary complex of one IGF peptide, one IGFBP-3 or IGFBP-5 protein, and an 86-kDa acid-labile subunit (ALS) (6–9).

The human ALS (hALS) complementary DNA (cDNA) sequence predicts a protein with 18–20 leucine-rich repeats of 24 amino acids (10); also present are 7 asparagine-linked glycosylation sites, which are important for binding of ALS to IGFBP-3 (11). Binding of the IGFBP-3/IGF-I complex with ALS to form the serum ternary complex greatly prolongs the circulating half-life of IGF-I (12). This is in part due to the decreased ability of ternary complexes to cross the capillary endothelial barrier; also, binding of ALS to IGFBP-3 or -5 may prevent proteases from binding to and degrading these IGFBPs, with subsequent release of IGFs to tissues (1, 2, 13–15). In either case, IGFs in ternary complexes are not readily bioavailable; whether these complexes serve primarily as an IGF reservoir or as a way to prevent unwanted insulin-like metabolic effects of IGF peptides (1, 2) is unclear.

ALS is expressed in a tissue-specific pattern, with synthesis confined almost exclusively to parenchymal cells of the postnatal liver (16, 17). GH treatment increases ALS protein levels in serum and in medium conditioned by primary rat hepatocytes *in vitro*; these increases result from the ability of GH to induce ALS messenger RNA (mRNA) levels in rat hepatocytes *in vitro* and *in vivo* (18–23). GH stimulation of ALS mRNA levels in liver of hypophysectomized rats is mediated at the level of ALS gene transcription (21).

The chromosomal genes for mouse ALS (mALS) and rat ALS (rALS) have been cloned (24, 25). They share a simple organization with the protein-coding and 3′-untranslated regions contained in two exons separated by a single intron, which is identically positioned in the ALS genes from these two species. The 5′-flanking sequences in these two ALS genes are also quite similar. In the mALS gene, approximately 2 kb of the 5′-flanking region demonstrated promoter activity when transfected into rat H4-II-E hepatoma cells or primary hepatocytes, and GH significantly increased promoter activity (21, 24, 26). GH responsiveness of the mALS promoter has been mapped to a single DNA motif resembling an interferon-γ-activated sequence-like element (GLE) (26). The present studies characterize the location and organization of the hALS chromosomal gene and show that the

Received August 2, 1999.
Address all correspondence and requests for reprints to: Dr. David R. Powell, Texas Children's Hospital, Feigin Center, MC# 3–2482, 6621 Fannin, Houston, Texas 77030. E-mail: dpowell@bcm.tmc.edu.
* This work was supported by NIH Grants RO1-DK-38773 (to D.R.P.) and RO1-DK-51624 (to Y.R.B.), and by the βσφ Research Fund, Houston City Council (to D.R.P.).

hALS and mALS promoter regions are homologous. These studies also demonstrate that the GH-responsive GLE is 100% conserved in and is able to confer GH stimulation to the hALS promoter.

Materials and Methods

General methods

Oligonucleotides used for sequencing, site-directed mutagenesis, and PCR amplifications were synthesized by either Ana-Gen Technologies (Palo Alto, CA) or the Child Health Research Center Core Facility at Baylor College of Medicine (Houston, TX). All native and mutant sequences and construct orientations were determined by DNA sequencing either using Sequenase (U.S. Biochemical Corp., Cleveland, OH) in the dideoxy chain termination method (27) or by dye terminator automated sequencing performed by the Child Health Research Center Core Facility using an ABI/Perkin-Elmer Corp. automated sequencer (Palo Alto, CA).

Isolation of hALS cDNA

One microgram of human liver total RNA (CLONTECH Laboratories, Inc., Palo Alto, CA) was reverse transcribed using random hexamer priming and SuperScript II (Life Technologies, Inc./BRL, Rockville, MD). This material served as template for oligonucleotides primers 5′-CTTCCTCAAGGACAACGG-3′ and 5′-TTCCTGAGGCTGAGGTAGC-3′ in a PCR amplification using *Taq* polymerase (Life Technologies, Inc./BRL), resulting in a 300-bp hALS DNA fragment spanning from 1245–1544 bp of the published hALS cDNA sequence (10). This amplicon was visualized by 8% PAGE, cut from the gel, eluted, ethanol precipitated, and then amplified in a second round of PCR. The purified DNA product served as template for ^{32}P labeling of this 300-bp fragment using the same oligonucleotide primers and PCR conditions; labeled DNA was then used to screen a Unizap human liver cDNA library (Stratagene, La Jolla, CA) as described previously (28). As no full-length hALS cDNA was isolated, a *Sma*I/*Bsu36*I fragment (1578–1898 bp of the published sequence) from a partial hALS cDNA was ^{32}P labeled and used to screen a human liver 5′-STRETCH PLUS cDNA library (CLONTECH Laboratories, Inc.). A 2-kb hALS cDNA was isolated and subcloned into pSP73 using *Eco*RI. This cDNA, spanning from 30–2039 bp of the hALS sequence, was subcloned into M13mp19 at the *Eco*RI site. Oligonucleotide 5′-TACCGAGCTCGAATTCCATGGCCCTGAGGAAAGGAGGCCTGGCCCTG GCGCTGCTGCTGCTGT-3′ was used to introduce the missing 29 bp of hALS coding sequence by site-directed mutagenesis using the Kunkel method, as described previously (29). The full-length hALS cDNA was subcloned into pBluescript II SK$^-$ (Stratagene) at the *Eco*RI site, creating phALS.

Isolation of a human ALS genomic clone

A 1.5-kb *Sac*II/*Eco*RI fragment spanning from 570-2039 bp of the hALS sequence was labeled with ^{32}P and used to screen a human placental genomic library constructed in phage EMBL 3 (CLONTECH Laboratories, Inc.) as described previously (30). A single λhALS clone was plaque purified, cleaved with several restriction endonucleases, and transferred to filters. The filters were prehybridized, hybridized, and autoradiographed as described previously (30), using both the 1.5-kb *Sac*II/*Eco*RI 3′-hALS cDNA fragment, and a 5′-*Eco*RI/*Sac*II fragment spanning from 1–575 bp of the hALS cDNA sequence, as ^{32}P-labeled probes. A 4.8-kb *Kpn*I/*Sac*II fragment of the λhALS clone, which hybridized with the 5′- but not the 3′-hALS cDNA probe, was then subcloned into pBluescript II SK$^-$ at *Kpn*I/*Sac*II, creating pg5hALS. Partial DNA sequencing of this fragment revealed that the 3′-end was identical to a region of the hALS cDNA extending to the *Sac*II site at bp 575 of the published hALS sequence (10). To further characterize the 3′-end of the hALS gene, the λhALS DNA was used as template for oligonucleotide primers 5′-CTCAACCTCGGCTGGAATAG-3′ and 5′-CGATTGCCTTTGCCTTTAATTG-3′, which were used to PCR amplify the 3′-region of the hALS gene spanning from to 523-1998 bp of the published hALS cDNA sequence. This PCR product was subcloned into pCR2.1 (Invitrogen, San Diego, CA), creating pg3hALS.

Fluorescence in situ hybridization

The 4.8-kb *Kpn*I/*Sac*II fragment of the λhALS clone was used to probe standard metaphase spreads obtained from the peripheral blood lymphocytes of a human male donor. Details of probe labeling, chromosome identification, fluorescence *in situ* hybridization procedures, and digital imaging and processing have been reported previously (31).

Plasmid construction

A 1.4-kb fragment containing the ALS promoter region was released from pg5hALS with *Sal*I (5′)/*Sph*I (3′), subcloned into M13mp19 at these sites, and sequenced. A 0.8-kb fragment containing the proximal hALS promoter was released from m13mp19 with *Nco*I (5′)/*Bam*HI (3′) and subcloned into the *Hin*dIII site 5′ to the luciferase reporter gene in pGL3-Basic (Promega Corp., Madison, WI), creating p755hALS. A GLE spanning from 667–675 bp 5′ to the hALS ATG translation start codon, which is 100% conserved in the mALS gene and confers GH stimulation to the mALS promoter (26), was mutated using oligonucleotide 5′-TGCAGCCCTGCCAG GCAACGTATCGTGAGGCTGGGGGCGGGGC-3′. The 0.8-kb hALS promoter fragment containing the mutated GLE was released from m13mp19 with *Nco*I (5′)/*Bam*HI (3′) and subcloned into the *Hin*dIII site in pGL3-Basic, creating p755hALSmGLE. The construction of p703WT, which contains the proximal 703 bp of the mALS promoter 5′ to the luciferase reporter gene in pGL3-Basic, has been described previously (26).

Cell culture and DNA transfection

Primary hepatocytes, isolated from male Sprague Dawley rats (250–300 g) by procedures approved by the Cornell University Institutional animal care and use committee, were plated in six-well plates at a density of 1×10^6 cells/well and maintained as described previously (26). Hepatocytes were washed twice with serum-free modified William's E medium (MWEM) and then transfected for 14 h with a 1-ml solution of serum-free MWEM containing 1.2 μg luciferase plasmid, 0.018 μg pCMV-SEAP, which controlled for transfection efficiency, and 15 μg lipofectin (Life Technologies, Inc./BRL). After transfection, cells were cultured for 48 h in MWEM in the presence or absence of 100 ng/ml bovine GH (bGH); for the first 24 h, MWEM was supplemented with Matrigel (Becton Dickinson and Co., Bedford, MA). At the end of this 48-h period, medium was assayed for luciferase activity as described previously (26) and for secreted alkaline phosphatase by chemiluminescence following the recommendations of the manufacturer (Tropix, Bedford, MA).

Expression of hALS

Plasmid pKG3226 contains the human β-actin promoter, simian virus 40 polyadenylation signal, and neomycin phosphotransferase resistance gene (32). Full-length hALS cDNA was released from phALS and subcloned into pKG3226 with *Eco*RI; the resulting expression vector, pKG3226/hALS, was stably transfected into Chinese hamster ovary (CHO)-K1 cells as described previously (14). Cells were incubated in serum-free McCoy's 5A medium, and the medium was then screened for hALS expression by immunoblot as described previously (14), using a 1:7500 dilution of goat antihuman ALS antibody (Diagnostics Systems Laboratories, Inc., Webster, TX). ALS partially purified from human serum on a hIGFBP-3 antibody column (14) was used as a positive control on the immunoblot.

Results

Isolation of a hALS cDNA

A nearly full-length hALS cDNA was isolated from a human liver cDNA library as described in *Materials and Methods*. It spanned from 30–2039 bp of the published hALS cDNA and was identical to the published sequence (10). After the missing 29 bp of 5′-hALS coding sequence were added by site-directed mutagenesis, the full-length hALS cDNA was inserted into eukaryotic expression vector pKG3226 and

transfected into CHO-K1 cells. Cells stably transfected with pKG3226/hALS expressed an approximately 85-kDa protein that comigrated with partially purified hALS and was recognized by hALS antiserum (Fig. 1); this protein was not expressed by cells transfected with pKG3226 alone (data not shown).

Isolation and characterization of the hALS gene

A single λhALS genomic clone was isolated by screening 3×10^5 plaques from a human placental genomic library using both 5'- and 3'-hALS cDNA probes. Southern blotting of this plaque-purified λhALS clone identified a 4.8-kb *Kpn*I/*Sac*II λhALS fragment, which was recognized by the 5'-, but not the 3'-, hALS cDNA probe. DNA sequencing confirmed that the 3'-end of this 4.8-kb genomic fragment was identical to a region of the hALS cDNA spanning from bp 17 to the *Sac*II site at bp 575 of the published hALS sequence (10).

Partial sequencing of the 4.8-kb *Kpn*I/*Sac*II genomic fragment revealed a single 1235-bp intron beginning 17 bp 3' to the ATG translation start codon. To determine whether additional 3'-introns were present in the hALS gene, oligonucleotide primers were designed to PCR amplify the region of the hALS cDNA that spans from 532-1998 bp and contains the polyadenylation signal. These primers amplified an identical 1466-bp PCR product when either λhALS or the hALS cDNA served as template, thus confirming the absence of additional introns in the hALS gene. The organization of the hALS gene is shown in Fig. 2.

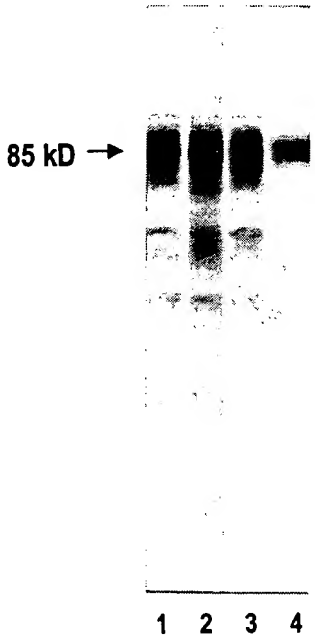

FIG. 1. hALS expression. A full-length hALS cDNA was placed in the eukaryotic expression vector pKG3226. This construct was then stably transfected into CHO-K1 cells. Conditioned medium from three independent clones (lanes 1–3) was screened for hALS expression by immunoblot using a goat anti-hALS antibody. ALS partially purified from human serum (lane 4) served as a positive control. The estimated size of hALS, in kilodaltons, is shown on the *left*.

Chromosomal localization of the hALS gene

Fluorescence *in situ* hybridization revealed that for all analyzed metaphases, only chromosome 16 hybridized with the 4.8-kb *Kpn*I/*Sac*II fragment of λhALS (Fig. 3). Q-banding placed the ALS gene on the short arm at p13.3 (data not shown).

hALS gene organization 5' to the ATG translation start codon

DNA sequencing of pg5hALS established the proximal 1398 bp of the hALS gene 5' to the ATG translation start codon. As shown in Fig. 4, this region is homologous to the comparable region of the mALS gene that contains the mALS promoter (24) and to the comparable region of the rALS gene (25). In particular, this region of the hALS gene contains the GLE 5'-TTCCTAGAA-3', spanning from −675 to −667 bp 5' to the ATG translation start codon, which is conserved in sequence and location in the mALS and rALS 5'-flanking regions. In the mALS gene, this GLE confers the stimulatory effect of GH on mALS promoter activity (26).

Identification of a GH-responsive element in the hALS promoter

To determine whether the hALS promoter is responsive to GH, the region spanning from −755 to −4 bp 5' to the hALS ATG translation start codon was placed 5' to the luciferase reporter gene, creating p755hALS. When primary rat hepatocytes were transiently transfected with p755hALS and incubated with or without 100 ng/ml bGH for 48 h, luciferase values rose 45% in the presence of bGH (Fig. 5). When hepatocytes were transiently transfected with p755hALSmGLE, which is identical to p755hALS except that the potentially GH-responsive GLE in the hALS promoter is replaced with the sequence 5'-ACGTATCGT-3', luciferase values were comparable in cells incubated with or without bGH. In hepatocytes transiently transfected with the positive control plasmid p703WT, which contains the proximal 703 bp of the mALS promoter 5' to the luciferase reporter gene, bGH stimulated p703WT activity by 167% (data not shown).

Discussion

The hALS gene contains two exons that span about 3.3 kb of DNA. The intron/exon splice junctions of the single hALS intron conform to consensus sequences derived from other vertebrate genes (33) and are identical in location to those of the rALS and mALS genes (24, 25). Although the single hALS intron is similar in size to the mALS and rALS introns, it shares only about 40% nucleotide identity with these rodent introns and contains no regions of high similarity that might suggest an important regulatory region. The hALS cDNA and gene exon sequences were identical to the published hALS cDNA sequence (10).

The hALS gene is located on chromosomal region 16p13.3 near the α-globin gene complex. Deletions of the 16p13.3 region are described in eight individuals with α-thalassemia and mild to moderate mental retardation (34). In these cases, α-thalassemia results from failure of the individual to inherit a normal α-globin allele from one parent. In addition to

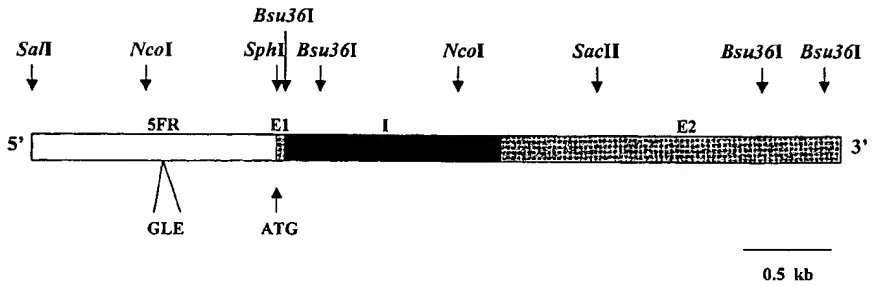

FIG. 2. hALS gene organization. This schematic diagram depicts the organization of the hALS chromosomal gene. The 5'-flanking region (5FR) is represented by the *white rectangle* to the *left* of the ATG translation start codon; the location of the GLE within this region is shown. Exons 1 and 2 (E1 and E2) are represented by *gray rectangles* to the *left* and *right* of the *black rectangle* that represents the single intron (I). *Arrows* indicate the locations of selected restriction sites. Nucleotide sequence of the chromosomal region depicted here has been submitted to GenBank/EMBL data bank with accession number AF192554.

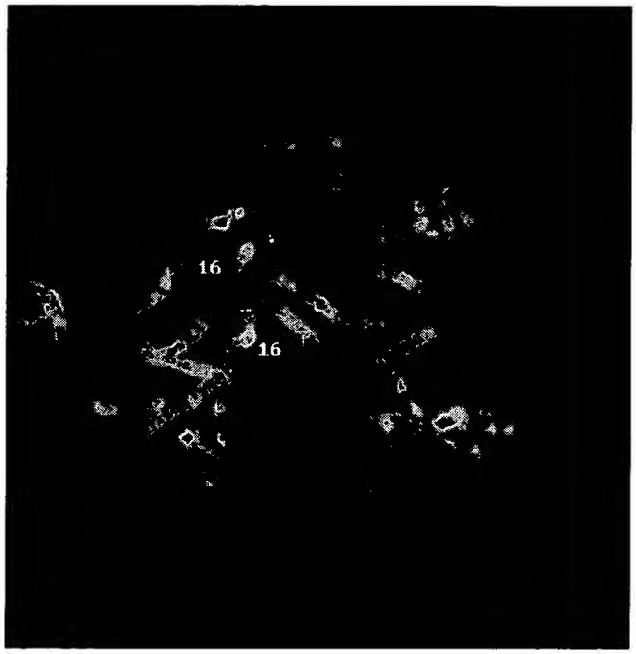

FIG. 3. Chromosomal localization of the hALS gene. Fluorescence hybridization of a 4.8-kb hALS genomic fragment to standard human metaphase chromosomes. Fluorescent signals can be seen on both chromatids of each chromosome 16.

α-thalassemia and mental retardation, a variety of dysmorphic features are also described in these individuals. In general, their clinical phenotype is nonspecific, and short stature was present in only two of the eight patients. In mice, growth failure occurs with targeted inactivation of both, but not one, of the ALS alleles (Boisclair, Y. R., unpublished results). Thus, growth failure is unlikely to be a part of the α-thalassemia/mental retardation phenotype, as these individuals have at least one ALS allele.

Although the rALS and mALS 5'-flanking regions share 87% nucleotide identity over the first 1318 bp of the mouse sequence, they apparently differ in the locations of their transcription start sites (24, 25). In the rALS gene, the major transcription start sites were located 447, 472, and 505 bp 5' to the ATG translation start codon. In contrast, the transcription start sites in the mALS gene clustered between 10–161 bp 5' to the ATG translation start codon, a region that is entirely contained within a known rALS cDNA sequence. This variability may be due to the fact that consensus TATA sequences, initiator sequences, or GC boxes, which serve to target the site of transcription initiation in most genes, are not conserved in the 5'-flanking regions of the ALS genes (24, 25). Transcription start sites were not mapped in the hALS gene due to lack of sufficient intact human liver RNA and to lack of human cell lines expressing ALS. However, comparing the known size of hALS mRNA transcripts in liver with the hALS cDNA sequence (10) suggests that hALS transcription start sites more closely approximate those in the mALS than the rALS gene.

Although rALS and mALS 5'-flanking regions are highly similar, they share only 51% and 47% nucleotide identity, respectively, with the proximal 1398-bp region of the hALS gene. However, the sequence spanning from −688 to −625 bp of the hALS 5'-flanking region is 81% and 77% conserved in the rALS and mALS 5'-flanking regions, respectively. The GLE that confers GH stimulation to the mALS promoter is present in this region of the mALS gene and is 100% conserved in the rALS and hALS genes (24–26), suggesting that this GLE participates in GH stimulation of hALS and rALS transcription. This hypothesis is supported by studies presented here, which show that GH does indeed stimulate the activity of the hALS promoter construct containing the native, but not the mutant, GLE. The effect of GH on mALS promoter activity appears to be mediated by members of the signal transducers and activators of transcription (STAT) protein family, STAT5a and STAT5b, which bind directly to this GLE in a GH-dependent fashion (26, 35). Considering that the GLE is 100% conserved in the hALS gene, it seems likely that STAT5a and STAT5b also confer GH stimulation to the hALS promoter. Although this hypothesis has not been tested, the data presented here provide strong evidence that GH uses a common mechanism to stimulate transcription of human and mouse ALS genes.

The significance of GH's ability to more potently stimulate the mALS than the hALS promoter is unclear. Certainly, the transcriptional activity of STAT5 isoforms can be affected by the nature of surrounding DNA sequence (36–38). This suggests that although the GLE is necessary for GH stimulation of hALS and mALS promoter activity, nearby DNA elements and the proteins that they bind modulate STAT5 transcriptional activation and account for the differences in GH-stim-

```
Human -1398  TGGGTCGACTCTAGGCCTCACTGGCCTAATACGACTCACTATAGGGAGCTCGAGGATCGTGGGTCTTTCTGGGTGTGGAC
Rat   -1295  A-A-G-TGGC-ACT-G-C-CGGA---CTGCTGAGGC-G-AC-TTT-CTG-GT-AAC-GC-CT--GGC------C-GA---
Mouse -1318  A-A-G-TGGCTACT-G-C-CG-A--TCTGCTGAGAC-G-AC-TTT-CTG-GT-AAC-GC-CT--GGC------C-GA---

Human -1318  TTTCTCCCCGTGGATCTATAATTTCTCAGAAGAGGAGCCGAGACGCCACCTGGAAAATGATCAATTCTCGATAAAGGGAA
Rat   -1215  -CC-------CA-------------------A--AG--T---GAATA-----------CC-T-GA-AT-GG-C--CATG
Mouse -1238  -CC-----T-CC-------------------A--AG--T---GAATA--A---------CC-T-GA-AT-GGGAG-CATG

Human -1238  GACGATGACAGTGGGCCGGCGGCTTCCCACAGCTCTGCACGGGGCCTGCCAGGTGAG.GCCGGAGCTGAGGATGGGGT..
Rat   -1135  -TG-GAAC--CA-TC--CTGAC-------G-C--GCCT-TTTCCT----TT-C-T-..-AGAT----TCCA---ATA-CC
Mouse -1158  -TG-GAGC--CA-.C--CTGAC-C-----G-C--GCCT-CTTCCT----TT-C-T-..-AGATG---CCCA---....TC

Human -1161  ..AGGGCCCTGGAAAACTGTGGTCTCCCCGAGCCCTGGCTGTGCCCCCGCCTGCACCCCTTCCGGCTGCTTCCCTGCACA
Rat   -1057  CCCA-T---CCT--G-AAC--TC--T--T--AA--CA-TAC----T-TC--G--C--T-......-C-TG-G-A-T-C--
Mouse -1085  CCCCAAG--CCTG-G-AGC--T---TT-T--AA--CA-CAC----TTGC-TG--C--T-......-C-TG-G-A-T-C-C

Human -1083  CTTTCC....CACTGCCGAGGGACTGGTGGGCAGGCCCCACTGACAGCCAGGGGAGGGGCCTGCAGGGACCTGGAGGGGA
Rat    -981  -CA---....--GG--AA------A-AGCTCA---.........---GGTTT-CT-A-CTT--A--T-T--A-C---TC-
Mouse -1009  -CA---CGGG--GG--AA------A-AGCTC.---.........---GACTG-C.......--G--T-T--GTC---TC-

Human -1007  GGGGCTGCACCTGCTCAGGCCCTGGGGGAGAGAGCGACTGGTTGGGAAGTGGTTTGAGCAGGTGGAGGGGAACCAGGGGC
Rat    -914  CA-.........CT-----------A----GA---.......--A--T----C-CCG-----GT---T---G------C-
Mouse  -946  CA-.........GTT----------A----GA---.......--A--T----C-CCG-----GT---T---G------C-

Human  -927  CTCTGGAGGCAGGTGGAGACGGAGCCCAGGGGTGGCCTTGGACATGGCTGGGCACACATTCCTGTGGCCTCTCTGCACAC
Rat    -849  AGG-T----GTCCCAC-TG-A-CC-T-CT-CC-CC--CA--GACA-CA--TTT--C-.........--GG---T-------
Mouse  -881  AGG-T----GCCCCAC-C-T--CC-T-CT-CC-CC--C---GGCA-CA--TTT--C-.........--GG---T-------

Human  -847  AAGCGGTGACTGTGGGGCG....GCCAGGCCCTCACCAGAGCCTTGGGGGACAGCAAGGGGCAGGTGAGGCTTGTCTGAG
Rat    -777  ---..........-AA-TA....-TT--------CAT-CT-TGGA--CC-GTG-A-GCT---.............-----C
Mouse  -809  ---..........-TA-TAGGTT-TT--------CAT-CT-TGGA--CCAG---A--CT---.............----CC

Human  -771  GCACAAGACCTGGCTCCCATGGCCAGGCCACCCCCACACACTCCCCGAGGATGGCGCAG..GGCAAAAGCAAACA....G
Rat    -723  C-T-C--G-T---G-G--CACTGTCTT--TG--AGC--GC--TT--T----G-A-TGT-CT--GT-G--------TGAG-
Mouse  -732  C-TG-G-CTGC--TG---CACTGTCTT--TG--A...A-C--TTT-T----G-A-TGT-CT--GG-G--------CGAG-

Human  -697  GCGCCTGCAGCCCTGCCAGGCATTCCTAGAAGAGG..CTGGGGGCGGGGCAGTGGCCAGACACCAGCGCCCCTGGGGTCA
Rat    -643  TA--TG-TG-----------TG-------------CC----A--A--------A--------T----TGA---A-....-
Mouse  -655  TA--TG-TG--T--------TG-------------CC-------A-----G--A-----C-GT----TGA---A-....-

Human  -619  CGGGAGAGGCAGGGTGAGCTTGGGCCCTGGACAGCACCGTGGGCCTGAAGCCCAAACGGCAGCTCCCACCCAGGCTGTAG
Rat    -567  G-TC-C---A---C----AC------T------AAC--C-----TG-G--G-AC--AT-GT-GG-----T------TA--
Mouse  -579  G-TC-C---A--AC----AC------T-A----AAC--C---A-TG-G--G-AC--AT-GT-GG-----TT----CTA--

Human  -539  GGAACCCCCGGGAGGGCGGGTGGATG..GCTGGGTGGGGCACGGGGAAGGG..GCGGCAGCCCCTTAGCCCCACACGGCT
Rat    -487  AA-C---AG-TT--ACA-CA---GA-AA-TACAAGCAA-GC-TTA-G-ACC..--AAA-CTG-T-A-C--TG----A---
Mouse  -499  AA-C---AG-TC--AGA-CA-TCGA-AA-TATA-GCAA-GC-TTA-G-AACAG-GCAA-CTGTT-A----TG---GA-TC

Human  -463  GGCTACTCAGGCCAGGGGTGGGCTCAAGACCCCAGGAGCTGCCCGAGGAGCCCCCTGCCCGCAACAGGACCTGGTAAAAC
Rat    -409  ATC-G-----TAT------.-AA-AGG-GGATTCCC--GAA---CG-AGCT-----CA--TTGT-T-A----AT-GGG-G
Mouse  -419  AT--G-T-C-TAT--A---.-AG-GGG-GGGTTCCC--GAA---CC-AGCT.---TCA--TTAG-T-A----AC-GGG-G

Human  -383  CCTGGGCTCCCCAAACCGGCCTCAAGCCCCAGCCCCTCCCAGGCTGCTGCAAGGGGTCCCCACCCCAACCTGGCTGCCCA
Rat    -330  A-A---TC---T-----TA---TG---A---.............A-C---T---ACTA-T---.............--A-
Mouse  -341  A-A----C---T-C---TA---TG---A---.............A-C-C-A-G-CCTA-T---.............--A-

Human  -303  GCCCTCCCCAGTCAGCCCTGCAGGGGGCCCGGGCACTGGCCGTCAGCCC...........CCAGGGCCGCATTACGGCTA
Rat    -276  ---TCTA--TCCA-CA--A-------TGG-TTTT-AA-T-.....T---...........TG----AGA-T---G---AG
Mouse  -287  ---TCTA--TCCA-CA--A----A--TGG-TTT--A-T--.....T---CCGCCCCTCAAT-CCC-AGA-T--GG---GG

Human  -234  CCTCGGGCCCCGACTGAAGCTCCCTCACCCCCTGCACTGTCTGCACCCCAGGGCTCCGGGCTGATGCCGGCCGCCCGGCA
Rat    -212  A-GAA-TG---CT-CCTT-----A--TG-A--CAGGAGAG----.............T-TC--GA-TA--T----A---
Mouse  -212  A-AAA-TG---CT-CCCT--A--AC--G-AT-CAG-GGAG--T-.............T-T---GA-TT-TT----A--C

Human  -154  CGAGGGGGTTAACAGAGCCAGGGCCAGCTGGGTTGGGCCAGCCAGGTGACAGGCGCGGGCTGCCCCGCAGTGGGGAAATC
Rat    -146  ----A-------AG----G--.......A-T--G----AG-G-CA-CTGAG---TGCCAGCTA-AG--------------
Mouse  -146  -T--A-------AG-------.......A-T--G----AG-G-CA-CTGAG---TGCCAGCTA-AG--------------

Human   -74  CAGAGGGCAGGGGTGGCCGGCACAGCAGACGTACCCTCCCTCGCTGCCTGCCTGCAGCCTGCCCTGCATGCAGG...ATG
Rat     -73  --C------...-CA---T-T-TT-T-----.G----TG---A----------------A--T---T-CAAG-AACA---
Mouse   -73  --CT-----...-CA---T-TGCT-T-----.G----TG---A-----------G---A--T---T-CAAG-AACA---
```

FIG. 4. Comparison of 5′-flanking regions among the human, rat, and mouse ALS genes. DNA sequence from the human ALS 5′-flanking region was aligned with homologous regions from rat (25) and mouse (24) ALS genes using the algorithm of Smith and Waterman (30). Gaps in any sequence are represented by a *dot*. Nucleotides conserved between the hALS sequence and either the rALS or mALS sequence are represented by a *dash*. Numbers on the *left* refer to the distance, in base pairs, 5′ (negative) to the A of the ATG translation start codon; for each ALS gene, this ATG codon is shown as the last 3 bp of sequence. The GH-responsive GLE spanning −675 to −667 bp of the hALS sequence is *underlined*.

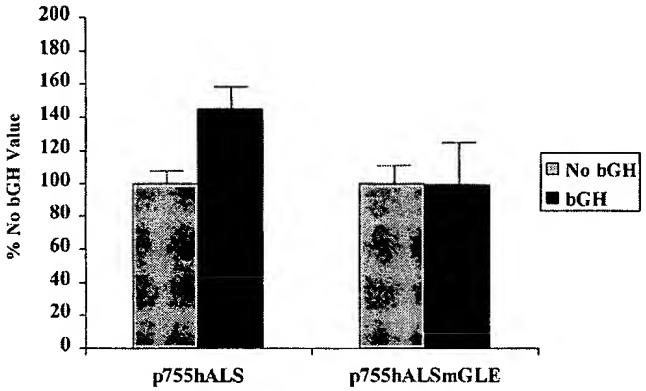

FIG. 5. Effect of GH on hALS promoter activity. DNA fragments spanning from −755 to −4 bp 5′ to the hALS ATG translation start codon and containing either the native or mutant GLE were placed 5′ to the luciferase reporter gene to create either p755hALS or p755hALSmGLE, respectively. These constructs were transiently transfected into primary rat hepatocytes and then cultured in serum-free medium in the presence or absence of 100 ng/ml bGH, as described in *Materials and Methods*. After 48 h, medium from each plate was assayed for luciferase activity. Activity of plasmids incubated without bGH were arbitrarily set at 100%; all values are presented as the mean ± SD and represent the results of two independent experiments performed in triplicate.

ulated activity of the mALS and hALS promoters. Based on these considerations, future studies will focus on identifying the proteins that interact to confer GH stimulation of hALS transcription.

References

1. **Jones JI, Clemmons DR** 1995 Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3–34
2. **Stewart CEH, Rotwein P** 1996 Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. Physiol Rev 76:1005–1026
3. **Rechler MM, Nissley SP** 1990 Peptide growth factors and their receptors. I. Insulin-like growth factors. In: Sporn MB, Roberts AB (eds) Handbood of Experimental Pharmacology. Springer-Verlag, Berlin, vol 95:263–267
4. **Shimasaki S, Shimonaka M, Zhang H-P, Ling N** 1991 Identification of five different insulin-like growth factor binding proteins (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human. J Biol Chem 266:10646–10653
5. **Shimasaki S, Gao L, Shimonaka M, Ling N** 1991 Isolation and molecular cloning of insulin-like growth factor binding protein-6. Mol Endocrinol 5:938–948
6. **Baxter RC, Martin JL** 1989 Binding proteins for the insulin-like growth factors: structure, regulation and function. Prog Growth Factor Res 1:49–68
7. **Martin JL, Baxter RC** 1986 Insulin-like growth factor binding proteins from human plasma. J Biol Chem 264:11843–11848
8. **Baxter RC, Martin JL, Beniac VA** 1989 High molecular weight insulin-like growth factor binding protein complex. J Biol Chem 264:11843–11848
9. **Twigg SM, Baxter RC** 1998 Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. J Biol Chem 273:6074–6079
10. **Leong SR, Baxter RC, Camerato T, Dai J, Wood WI** 1992 Structure and functional expression of the acid-labile subunit of the insulin-like growth factor binding protein complex. Mol Endocrinol 6:870–876
11. **Janosi JB, Firth SM, Bond JJ, Baxter RC, Delhanty PJ** 1999 *N*-Linked glycosylation and sialylation of the acid labile subunit. Role in complex formation with insulin-like growth factor (IGF) binding protein-3 and the IGFs. J Biol Chem 274:5292–5298
12. **Lewitt MS, Saunders H, Phuyal JL, Baxter RC** 1994 Complex formation by human insulin-like growth factor binding protein-3 and human acid-labile subunit in growth hormone-deficient rats. Endocrinology 134:2404–2409
13. **Campbell PG, Andress DL** 1997 The heparin binding region of insulin-like growth factor binding protein-5 (IGFBP-5) regulates plasmin degradation of IGFBP-5. Am J Physiol 273:E996–E1004
14. **Campbell PG, Durham SK, Suwanichkul A, Hayes JD, Powell DR** 1998 Plasminogen binds the heparin binding domain of insulin-like growth factor binding protein-3. Am J Physiol 275:E321–E331
15. **Durham SK, Suwanichkul A, Hayes JD, Herington A, Powell DR, Campbell PG** 1999 The heparin binding domain of insulin-like growth factor binding protein-3 (IGFBP-3) increases susceptibility of IGFBP-3 to proteolysis. Horm Metab Res 31:216–225
16. **Chin E, Zhou J, Dai J, Baxter RC, Bondy CA** 1994 Cellular localization and regulation of gene expression for components of the insulin-like growth factor binding protein complex. Endocrinology 134:2498–2504
17. **Dai J, Baxter RC** 1994 Regulation *in vivo* of the acid labile subunit of the rat serum insulin-like growth factor binding protein complex. Endocrinology 135:2335–2341
18. **Gargosky SE, Tapanainen P, Rosenfeld RG** 1994 Administration of growth hormone (GH), but not insulin-like growth factor-I (IGF-I), by continuous infusion can induce the formation of the 150-kilodalton IGF-binding protein complex in GH-deficient rats. Endocrinology 134:2267–2276
19. **Zapf J, Hauri C, Waldvogel M, Futo E, Hasler H, Binz K, Guler HP, Schmid C, Froesch ER** 1989 Recombinant human insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats. Proc Natl Acad Sci USA 86:3813–3817
20. **Baxter RC, Dai J** 1994 Purification and characterization of the acid labile subunit of rat serum insulin-like growth factor binding protein complex. Endocrinology 134:848–852
21. **Ooi GT, Cohen FJ, Tseng LY-H, Rechler MM, Boisclair YR** 1997 Growth hormone stimulates transcription of the gene encoding the acid labile subunit (ALS) of the circulating insulin-like growth factor binding protein complex and ALS promoter activity in rat liver. Mol Endocrinol 11:997–1007
22. **Dai J, Scott CD, Baxter RC** 1994 Regulation of the acid labile subunit of the insulin-like growth factor complex in cultured rat hepatocytes. Endocrinology 135:1066–1072
23. **Powell DR, Liu F, Baker BK, Hintz RL, Durham SK, Kale A, Brewer ED, Frane JW, Tonshoff B, Mehls O, Wingen A, Watkins SL, Hogg RJ, Lee PDK** 1997 Insulin-like growth factor binding protein-6 levels are elevated in serum of children with chronic renal failure. J Clin Endocrinol Metab 82:2978–2984
24. **Boisclair YR, Seto D, Hsieh S, Hurst KR, Ooi GT** 1996 Organization and chromosomal localization of the gene encoding the mouse acid labile subunit of the insulin-like growth factor binding complex. Proc Natl Acad Sci USA 93:10028–10033
25. **Delhanty PJ, Baxter RC** 1997 Cloning and characterization of the rat gene for the acid labile subunit of the insulin-like growth factor binding protein complex. J Mol Endocrinol 19:267–277
26. **Ooi GT, Hurst KR, Poy MN, Rechler MM, Boisclair YR** 1998 Binding of STAT5a and STAT5b to a single element resembling a γ-interferon-activated sequence mediates the growth hormone induction of the mouse acid labile subunit promoter in liver cells. Mol Endocrinol 12:675–687
27. **Suwanichkul A, Cubbage ML, Powell DR** 1990 The promoter of the human gene for insulin-like growth factor binding protein-1: basal promoter activity in HEP G2 cells depends upon liver factor B-1. J Biol Chem 265:21185–21193
28. **Allander SV, Coleman ME, Luthman H, Powell DR** 1997 Chicken insulin-like growth factor binding protein (IGFBP)-5: conservation of IGFBP-5 structure and expression during evolution. Comp Biochem Physiol 116B:477–483
29. **Allander SV, Durham SK, Scheimann AO, Wasserman RM, Suwanichkul A, Powell DR** 1997 HMGI/Y and HNF3 proteins bind the insulin response element of the IGFBP-1 promoter. Endocrinology 138:4291–4300
30. **Allander SV, Larsson C, Ehrenborg E, Suwanichkul A, Weber G, Morris SL, Bajalica S, Kiefer MC, Luthman H, Powell DR** 1994 Characterization of the chromosomal gene and promoter for human insulin-like growth factor binding protein-5. J Biol Chem 269:10891–10898
31. **Abu-Elheiga L, Almarza-Ortega DB, Baldini A, Wakil SJ** 1997 Human acetyl-CoA carboxylase 2. Molecular cloning, characterization, chromosomal mapping and evidence for two isoforms. J Biol Chem 272:10669–10677
32. **Spratt SK, Tatsuno GP, Yamanaka MK, Ark BC, Detmer J, Mascarenhas D, Flynn J, Talkington-Verser C, Spencer EM** 1990 Cloning and expression of human insulin-like growth factor binding protein 3. Growth Factors 3:63–72
33. **Green MR** 1991 Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. Annu Rev Cell Biol 7:559–599
34. **Wilkie AOM, Buckle VJ, Harris PC, Lamb J, Barton NJ, Reeders ST, Lindenbaum RH, Nicholls RD, Barrow M, Bethlenfalvay NC, Hutz MH, Tolmie JL, Weatherall DJ, Higgs DR** 1990 Clinical features and molecular analysis of the α thalassemia/mental retardation syndromes. I. Cases due to deletions involving chromosome band 16p13.3. Am J Hum Genet 46:1112–1126
35. **Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN** 1998 Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841–850
36. **Xu X, Sun Y-L, Hoey T** 1996 Cooperative DNA binding and sequence-selective recognition conferred by the Stat amino-terminal domain. Science 273:794–797
37. **Stocklin E, Wissler M, Gouilleux F, Groner B** 1996 Functional interactions between Stat5 and the glucocorticoid receptor. Nature 383:726–728
38. **Meton I, Boot EP, Sussenbach JS, Steenbergh PH** 1999 Growth hormone induces insulin-like growth factor-I gene transcription by a synergistic action of STAT5 and HNF-1α. FEBS Lett 444:155–159

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 274, No. 9, Issue of February 26, pp. 5292–5298, 1999
Printed in U.S.A.

N-Linked Glycosylation and Sialylation of the Acid-labile Subunit

ROLE IN COMPLEX FORMATION WITH INSULIN-LIKE GROWTH FACTOR (IGF)-BINDING PROTEIN-3 AND THE IGFs*

(Received for publication, April 29, 1998, and in revised form, November 20, 1998)

Jackie B. M. Janosi, Sue M. Firth, Judy J. Bond, Robert C. Baxter, and Patric J. D. Delhanty‡

From the Kolling Institute of Medical Research, University of Sydney, Royal North Shore Hospital, St Leonards, New South Wales 2065, Australia

Over 75% of the circulating insulin-like growth factors (IGF-I and -II) are bound in 140-kDa ternary complexes with IGF-binding protein-3 (IGFBP-3) and the 84–86-kDa acid-labile subunit (ALS), a glycoprotein containing 20 kDa of carbohydrate. The ternary complexes regulate IGF availability to the tissues. Since interactions of glycoproteins can be influenced by their glycan moieties, this study aimed to determine the role of ALS glycosylation in ternary complex formation. Complete deglycosylation abolished the ability of ALS to associate with IGFBP-3. To examine this further, seven recombinant ALS mutants each lacking one of the seven glycan attachment sites were expressed in CHO cells. All the mutants bound IGFBP-3, demonstrating that this interaction is not dependent on any single glycan chain. Enzymatic desialylation of ALS caused a shift in isoelectric point from 4.5 toward 7, demonstrating a substantial contribution of anionic charge by sialic acid. Ionic interactions are known to be involved in the association between ALS and IGFBP-3. Desialylation reduced the affinity of ALS for IGFBP-3·IGF complexes by 50–80%. Since serum protein glycosylation is often modified in disease states, the dependence of IGF ternary complex formation on the glycosylation state of ALS suggests a novel mechanism for regulation of IGF bioavailability.

Insulin-like growth factors (IGF)[1] I and II are peptide hormones that regulate the differentiation and proliferation of a large number of cell types and also have a role in glucose homeostasis (1). At least 75% of the total circulating IGFs are carried in 130–150-kDa ternary complexes containing IGF-binding protein-3 (IGFBP-3) (2) and an 85-kDa glycoprotein, the acid-labile subunit (ALS) (3). Recently, IGFBP-5 was also found to form a similar ternary complex with the IGFs and ALS in serum (4). It is thought that the size of the ternary complex restricts the passage of IGFs to target cells, while free IGFs, or IGFs in binary complexes with IGFBPs, can cross the capillary endothelial barrier. Therefore, ALS regulates the hypoglycemic and mitogenic potential of the circulating IGFs via the formation of the ternary complexes. Furthermore, ALS plays a vital role in maintaining a circulating store of the IGFs, IGFBP-3, and possibly IGFBP-5, by significantly increasing their serum half-lives (5, 6).

Despite the importance of the ternary complexes in regulating serum IGF bioactivity, nothing is known about the structural aspects of ALS that enable it to interact with IGFBPs. There are two features of ALS structure that may play a part. First, the protein backbone of ALS is made up of repeating blocks each containing 24 amino acids, of which 6 are typically leucine residues. This places ALS in the leucine-rich repeat superfamily of proteins (7), all of which are involved in protein-protein interactions (8). Second, serum ALS is heavily and heterogeneously glycosylated with *N*-linked glycan chains (3), and glycosylation is known to influence the interactions of many proteins.

Electrophoretic studies have shown that human ALS circulates as two glycoforms. Serum-purified ALS displays a characteristic doublet on SDS-PAGE at 84–86 kDa, which is reduced to a single band of less than 70 kDa after enzymatic removal of the *N*-linked sugars (3). There are seven consensus *N*-linked sugar attachment sites within the amino acid sequence of ALS that are conserved between primate and rodent (7, 9–11). One site occurs almost in the center of the sequence, and a cluster of three sites is found toward each terminus. Between six and seven bands are observed upon partial deglycosylation of ALS derived from serum, suggesting that multiple sites are used (12).

Although there are no studies that directly identify physical features of ALS involved in ternary complex formation, there is evidence that charge-charge interaction exists between ALS and the IGF·IGFBP-3 binary complex. Polyanions, polycations, and increasing ionic strength all decrease the affinity of ALS for IGFBP-3 (13, 14). Recently we have shown that the removal of basic residues in the carboxyl-terminal region of IGFBP-3 decreased its affinity for ALS by 90%, indicating the importance of positive charge in this region (15). From these observations, it is likely that negative charges on ALS may be involved in the interaction. At physiological pH, ALS binds tightly to weak anion exchange columns, indicating that it has a net negative charge (3).

Since carbohydrates are a potential source of negative charge in glycoproteins, as well as being involved in protein interactions, we have investigated whether *N*-linked sugars on ALS play a role in the formation of ternary complexes between IGFs, IGFBP-3, and ALS.

* This work was supported by Grants 940447 and 960875 from the National Health and Medical Research Council, Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. E-mail: delhanty@med.usyd.edu.au.

[1] The abbreviations used are: IGF, insulin-like growth factor; ALS, acid-labile subunit; IGFBP, insulin-like growth factor-binding protein; Endo F, endo-β-*N*-acetylglucosaminidase; PNGase F, peptide-*N*⁴-(acetyl-β-glucosaminyl)-asparagine amidase; NANase III, *N*-acetylneuraminidase III; *O*-glycosidase, endo-α-*N*-acetylgalactosaminidase; DANA, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; α-MEM, α-modified Eagle's medium; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

EXPERIMENTAL PROCEDURES

Reagents

Preparations of natural human ALS, human IGFBP-3, and rabbit antiserum against IGFBP-3 were similar to those used in previous studies (3, 16). ALS was radioiodinated and purified by ion-exchange chromatography as described previously (17). IGF-I (Genentech, San Francisco, CA) was iodinated and cross-linked to IGFBP-3 as in previous studies (18). Restriction enzymes and materials for site-directed mutagenesis were from Promega Corp. (Madison, WI). Bovine serum albumin (BSA; radioimmunoassay grade, fraction V), γ-globulin, hexadimethrine bromide (Polybrene), dexamethasone, hypoxanthine, xanthine, thymidine, and mycophenolic acid were purchased from Sigma. Aminopterin was obtained from Life Technologies Inc.. Nucleoside-free α-modified Eagle's medium (α-MEM) and fetal calf serum were from Cytosystems (New South Wales, Australia). Centricon 30 microconcentrators were obtained from Amicon (Beverly, MA). *n*-Octyl glucoside, *O*-glycosidase (endo-α-*N*-acetylgalactosaminidase), and peptide-*N*-glycosidase F (PNGase F; peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase) were obtained from Boehringer Mannheim. *N*-Acetylneuraminidase III (NANase III) was from Glyko (Novato, CA). Endo-β-*N*-acetylglucosaminidase (Endo F containing <0.1% PNGase F; catalog no. 324706), 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA), and the sialic acid-specific lectin from *Tritichomonas mobilensis* were purchased from Calbiochem (La Jolla, CA).

Enzymatic Deglycosylation and Desialylation of ALS

Characterization of ALS Glycosylation—PNGase F (5 units) and NANase III (25 milliunits) were used to remove *N*-linked sugars and sialic acids, respectively. Reactions contained [^{125}I]ALS (4×10^5 cpm, ~40 ng), 50 mM sodium phosphate buffer (pH 6.5), and 0.1% (w/v) *n*-octyl glucoside, and were incubated at 37 °C for 16 h. Identical reactions were set up without enzyme as controls. To investigate *O*-glycosylation, [^{125}I]ALS (~40 ng) was treated at 37 °C with 5 units of PNGase F with the addition of 12.5 milliunits of NANase III after 8 h, then 2 milliunits of *O*-glycosidase 3 h later. The mixture was subsequently incubated for an additional 12–16 h at 37 °C.

Enzymatic Deglycosylation for IGFBP-3 Binding Studies—A series of increasingly deglycosylated [^{125}I]ALS preparations was generated by incubation with 2.3, 11.7, 29.3, and 58.6 milliunits of Endo F/ng of [^{125}I]ALS in 50 mM sodium phosphate buffer, pH 6.5, containing 0.0005% (w/v) *n*-octyl glucoside, for 16 h at 37 °C. For complete deglycosylation, [^{125}I]ALS was treated with 640 milliunits of Endo F/ng of [^{125}I]ALS in 0.0075% (w/v) *n*-octyl glucoside.

Desialylated [^{125}I]ALS was prepared using 0.035 milliunits of NANase III/ng of [^{125}I]ALS in 50 mM sodium phosphate buffer (pH 6.5) at 37 °C for 16 h. In experiments where DANA was used, it was added at 1 nmol/milliunit NANase III to specifically inhibit the sialidase activity of NANase III.

Site-directed Mutagenesis of ALS—The ALS cDNA was generated by reverse transcriptase-polymerase chain reaction from normal human liver total RNA as described previously (10). This cDNA was inserted into the *Bam*HI/*Xba*I site of pSELECT (Promega Corp.) for mutagenesis according to the manufacturer's recommended protocol. Mutagenic deoxyoligonucleotides were used to generate seven different ALS cDNAs, each containing Asn to Ala codon substitutions at one of the seven consensus *N*-glycan linkage sites (Asn^{37} → Ala, 5′-CTGCAGCTCCAGGgcCCTCACGCGCCTG-3′; Asn^{58} → Ala, 5′-GCTGGACGGCAACgcCCTCTCGTCCGTC-3′; Asn^{69} → Ala, 5′-GGCAGCCTTCCAGgcCCTCTCCAGCCTG-3′; Asn^{341} → Ala, 5′-CGTGGCGGTCATGgcCCTCTCTGGGAAC-3′; Asn^{448} → Ala, 5′-CCTCAGCCTCAGGgcCAACTCACTGCGG-3′; Asn^{527} → Ala, 5′-CTTCGCCCTGCAGgcCCCCAGTGCTGTG-3′; Asn^{553} → Ala, 5′-CCCGCGTACACCTACAACgcCATCACCTG-3′; substituted nucleotides are shown in lowercase). Oligonucleotides were synthesized using an Oligo 1000 DNA Synthesizer (Beckman, Palo Alto, CA). The mutations were confirmed by sequencing (T7 sequencing kit; Amersham Pharmacia Biotech), and then the cDNAs were excised from pSELECT and inserted into the *NheI*/*SalI* site of pMSG (Amersham Pharmacia Biotech), an expression vector that contains the constitutively active and glucocorticoid-inducible murine mammary tumor virus promoter.

Cell Culture and Transfections—Chinese hamster ovary (CHO) cells were transfected with either wild-type or mutant ALS expression constructs, or pMSG alone using the Polybrene/Me_2SO technique (19). pMSG contains the guanine phosphoribosyltransferase gene, which confers resistance to mycophenolic acid. Stable transfectants were selected for 3 weeks in α-MEM supplemented with 10% fetal calf serum, 25 μg/ml mycophenolic acid, 2 μg/ml aminopterin, 250 μg/ml xanthine, 15 μg/ml hypoxanthine, and 10 μg/ml thymidine. Some ALS transfectants were cultured from single foci to produce clonal lines. Flasks of stably transfected cells were grown to confluence in α-MEM supplemented with 10% fetal calf serum, then the medium was changed to α-MEM supplemented with 10 μM dexamethasone. After 3–4 days, the conditioned medium was collected and cell debris was removed by centrifugation. Supernatants were then concentrated and equilibrated into 50 mM sodium phosphate buffer (pH 6.5) using Centricon 30 microconcentrators. Conditioned media from CHO cells transfected with pMSG alone were concentrated to the same extent as media containing the lowest concentration of mutant ALS, and used as controls.

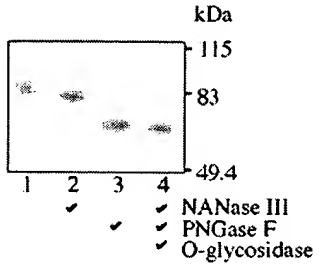

FIG. 1. **SDS-PAGE analysis of ALS after treatment with various glycosidases.** Preparations of [^{125}I]ALS were incubated with the glycosidases indicated (see "Experimental Procedures") and electrophoretically separated by nonreducing SDS-PAGE. Radiolabeled proteins were detected by autoradiography. The molecular mass markers are shown to the *right*.

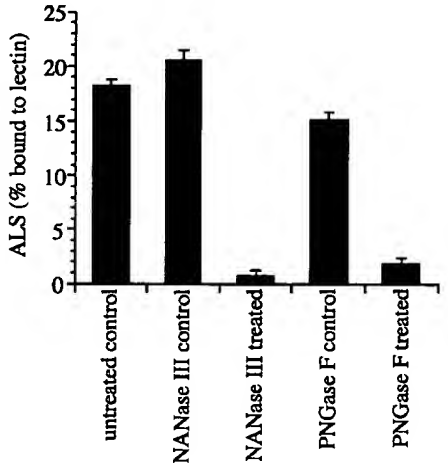

FIG. 2. **The sialic acids of ALS removed by NANase III treatment are on the *N*-linked sugars of ALS.** Preparations of [^{125}I]ALS in 50 mM phosphate buffer, NANase buffer without or with NANase III and PNGase F buffer without or with PNGase F were incubated overnight at 37 °C. The treated [^{125}I]ALS preparations were then assayed for binding to a sialic acid-specific lectin. All data were corrected for nonspecific binding. The data represent means ± S.E. of three separate experiments.

Electrophoretic Analyses

SDS-PAGE Analysis—Radioiodinated ALS (5,000 cpm, ~0.5 ng) in Laemmli buffer was loaded, without heat treatment, onto 7.5% Ready gels (Bio-Rad) and electrophoretically separated under nonreducing conditions. The gels were then dried and exposed to Hyperfilm MP (Amersham, Bucks, UK) overnight at −80 °C.

Isoelectric Focusing of ALS—Preparations of [^{125}I]ALS (10,000 cpm, ~1 ng), untreated or treated with NANase III, were added to sample buffer containing 4% (w/v) CHAPS (BDH Ltd, Poole, UK), 100 mM Tris-HCl, pH 7.2, and 2% (v/v) ampholytes (Pharmalyte 3–10, Amersham Pharmacia Biotech). Samples were loaded onto the alkaline end of an immobilized pH gradient strip (pH 3–10, Amersham Pharmacia Biotech) that had been rehydrated overnight in 0.5% (w/v) CHAPS, 10 mM dithiothreitol, 6 M urea, and 2% (v/v) Pharmalyte 3-10. Isoelectric focusing was performed in a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech). A broad pI isoelectric focusing calibration kit (pH 3–10; Amersham Pharmacia Biotech) was run in parallel with the

ALS samples. The theoretical pI for the human ALS protein backbone was calculated using the program ISOELECTRIC from the Genetics Computer Group, Inc. (Madison, WI).

Binding Assays

Gel Filtration Studies with Mutated ALS—Size fractionation chromatography was used to determine whether the seven site-directed mutant recombinant ALS species lacking individual consensus *N*-glycan linkage sites were able to form ternary complexes (18). Briefly, cross-linked [^{125}I]IGF-I·IGFBP-3 (1×10^5 cpm) was incubated for 30 min at 25 °C with conditioned medium containing 150 ng of mutant ALS equilibrated in 50 mM sodium phosphate buffer (pH 6.5) containing 1% (w/v) BSA, in a total volume of 200 μl. The mixture was then injected into a Superose-12 column (Amersham Pharmacia Biotech), and 0.5-ml fractions of eluate were collected at a flow rate of 1 ml/min. The degree of conversion from binary to ternary complex was evaluated by the shift of radioactivity from fractions corresponding to 50 kDa to those corresponding to 140 kDa (18).

Lectin Solution Binding Assay—A lectin solution binding assay was used to indicate the presence or absence of sialic acids on [^{125}I]ALS after enzymatic desialylation with NANase III and deglycosylation with PNGase F as described above. Identical reactions were set up without enzymes as controls. The assay was modified from that of Abidi *et al.* (20). Briefly, approximately 35,000 cpm [^{125}I]ALS (~3.5 ng), either treated with enzyme or untreated control, was incubated for 1 h with 2 μg of the sialic acid-specific lectin from *T. mobilensis* in 50 mM sodium phosphate buffer with 0.01% (w/v) BSA, pH 6.5 at 22 °C (final volume 100 μl). [^{125}I]ALS complexed to the lectin was then precipitated using γ-globulin (35 μl) and 6% polyethylene glycol (1 ml) for 10 min at 4 °C. Both BSA and γ-globulin were acid-hydrolyzed and dialyzed against water to remove contaminating sialic acids (21). The tubes were then spun at 3500 rpm in a swing bucket centrifuge for 10 min at 22 °C. The radioactive pellet in each tube was measured as a percentage of the total radioactivity added and these data were used to generate histograms. Nonspecific binding was determined to be the radioactivity measured when no lectin was added to the tube during the 1-h incubation. Control and desialylated forms of [^{125}I]ALS gave nonspecific binding ranging from 10% to 14% of total, and the PNGase F deglycosylated forms of [^{125}I]ALS gave approximately 28% of total.

Solution Binding Assay and Scatchard Analysis—Solution binding assays were carried out as described previously (16). Briefly, 10,000 cpm [^{125}I]ALS, either treated with enzyme or untreated control, was incubated for 2 h with 10 ng of IGF-I or -II and a range from 0 to 10 ng of IGFBP-3 in 50 mM sodium phosphate buffer, pH 6.5, at 22 °C (final volume 0.3 ml). ALS complexed to IGFBP-3 was then precipitated using IGFBP-3 antiserum. The radioactivity in each tube was measured as a percentage of the total radioactivity added, and these data were used to generate binding curves. Endo F (58.3 milliunits/ng ALS) was added to a control untreated [^{125}I]ALS preparation during the 2-h incubation to ensure that the presence of the enzyme did not adversely affect complex formation. Nonspecific binding was calculated as the percentage of radioactivity present after precipitation when there was no IGFBP-3 in the reaction mixture. For the Endo F deglycosylation experiments, nonspecific binding ranged from 3% to 18% of total for the partially deglycosylated forms and was approximately 30% of total for the fully deglycosylated forms. For the the NANase III binding curves, nonspecific binding was between 3% and 8% of the total. Scatchard analysis was carried out as described previously (16), except that IGF-I, IGF-II, and IGFBP-3 were held constant at 1 ng/0.3 ml. ALS was added over the range of 0–200 ng/0.3 ml.

Statistical Analyses—Binding curve data were analyzed by repeated measures analysis of variance, followed by Fisher's protected least significant difference test, using Statview 4.02 (Abacus Concepts Inc., Berkeley, CA). The value was considered significant if the p value was less than 0.05.

RESULTS

Glycosidase Characterization of ALS Carbohydrates—ALS is estimated to have ~20 kDa of *N*-linked carbohydrate, but the possible presence of *O*-linked sugars has not been investigated.

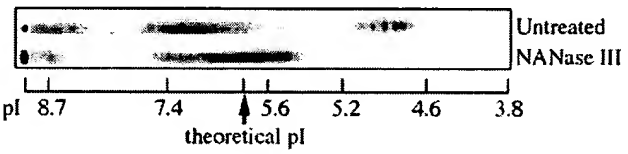

FIG. 3. **Isoelectric focusing profiles of native and NANase III-treated ALS.** Untreated and NANase III-treated [^{125}I]ALS were loaded at the alkaline ends (~pH 9) of immobilized pH gradient strips (pH 3–10) and focused electrophoretically (see "Experimental Procedures"). The relative positions of pI standards are indicated. The theoretical pI of the core ALS protein is indicated by an *arrow*.

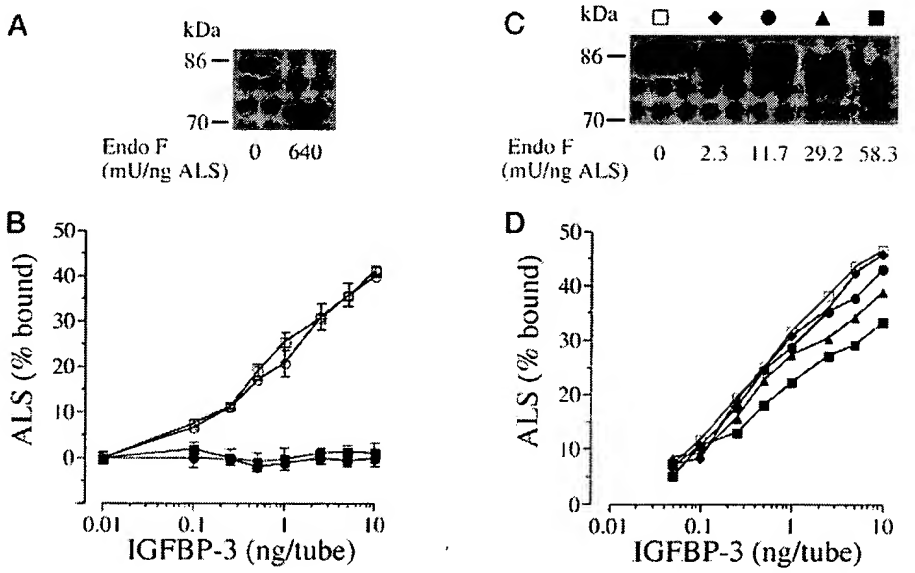

FIG. 4. **Partial or complete enzymatic deglycosylation reduces or abolishes, respectively, the ability of ALS to bind to IGFBP-3.** Preparations of [^{125}I]ALS were treated with Endo F for 16 h at 37 °C, subjected to nonreducing SDS-PAGE (*panels A* and *C*), and assayed for binding to increasing amounts of IGFBP-3 in the presence of 10 ng of IGF (*panels B* and *D*). *Panel A*, samples of untreated and completely deglycosylated [^{125}I]ALS subjected to SDS-PAGE and then detected using autoradiography. *Panel B*, binding curves of untreated (*open symbols*) and deglycosylated (*closed symbols*) ALS to IGFBP-3 in the presence of either IGF-I (*squares*) or IGF-II (*circles*). Deglycosylation of the [^{125}I]ALS used in these experiments was confirmed by SDS-PAGE (see *panel A*). Data shown are means ± S.E. of three separate experiments. *Panel C*, samples of [^{125}I]ALS partially deglycosylated with increasing concentrations of Endo F, subjected to SDS-PAGE, then detected by autoradiography. *Panel D*, binding of the natural and partially deglycosylated ALS samples shown in *panel C* to IGFBP-3·IGF-I complexes (□, ◆, ●, ▲, and ■ represent 0, 2.3, 11.7, 29.2, and 58.3 milliunits of Endo F/ng of ALS, respectively). Similar binding curves were seen in two independent experiments.

To address this, [^{125}I]ALS was treated with NANase III, which removes sialic acid, and *O*-glycosidase, which hydrolyzes some of the common core sugars of *O*-linked glycans. SDS-PAGE analysis was then used to identify shifts in the apparent size of treated ALS. Untreated [^{125}I]ALS (Fig. 1, *lane 1*) appears as a single band of approximately 85 kDa. The diffuse nature of the band may be explained by poor resolution of the 84–86-kDa doublet. The apparent size of ALS after NANase III treatment (*lane 2*) was decreased compared with untreated ALS. The approximate size difference is 2–3 kDa, consistent with the removal of 5–15 sialic acid moieties. ALS was also treated with PNGase F, an effective amidase that cleaves all types of *N*-linked sugars, to show the previously reported size shift to approximately 68–70 kDa (*lane 3*). This apparent molecular mass is very close to the predicted value of 66 kDa for the amino acid backbone of ALS. However, there is no difference in apparent size between ALS treated with PNGase F alone (*lane 3*) and ALS treated with PNGase F, NANase III and *O*-glycosidase (*lane 4*). These results suggest that ALS carries sialic acids that are predominantly or entirely attached to the *N*-linked sugar chains.

However, it is possible that, following *O*-glycosidase treatment, electrophoresis did not resolve the small shift in size predicted if *O*-linked sugars are a minor component of ALS carbohydrate. To investigate this further, we used a lectin binding assay (Fig. 2) to determine whether the sialic acids present on ALS could be accounted for by the *N*-linked sugars alone. A sialic acid-specific lectin derived from *T. mobilensis* was used to precipitate [^{125}I]ALS, which was either untreated or had been desialylated with NANase III or deglycosylated with PNGase F. Using this assay, we found that desialylation and deglycosylation caused 96 ± 4% and 88 ± 5% loss of binding, respectively. Since there was no significant difference between these values ($p = 0.1$, t test), we conclude that all of the sialic acids removed by NANase III treatment are derived from *N*-linked sugars on ALS.

Charge Contribution of the Sialic Acids to ALS—We used isolectric focusing to establish the net negative charge that the sialic acids would contribute to ALS. Untreated [^{125}I]ALS and NANase III-treated [^{125}I]ALS were separated by charge on immobilized pH gradient strips and visualized by autoradiography (Fig. 3). Untreated ALS was found to have at least six distinctly charged isoforms in the pI range of 4.5–5.2. Although the NANase III-treated ALS was less well resolved than the control, it is clear that the pI of the desialylated [^{125}I]ALS had shifted, as expected, toward a more neutral pI value of approximately 5.5–7.0. Furthermore, the distinct series of bands observed in the control disappeared after NANase III treatment. Therefore, the six distinct isoforms of ALS are consistent with a series of differentially sialylated ALS molecules.

Complete Deglycosylation of ALS Abolishes Ternary Complex Formation—In order to identify a potential biological role for ALS glycosylation, we investigated the effect that *N*-linked deglycosylation of ALS has on the formation of ternary complexes between the IGFs, IGFBP-3, and ALS. Enzymatic methods were used to deglycosylate serum-derived ALS. After radiolabeling, [^{125}I]ALS was treated with Endo F, an endoglycosidase mixture able to remove most types of *N*-linked sugars. The preparation of Endo-F used contained only trace levels of the asparagine amidase PNGase F. This was to limit the potentially confounding effects of converting the carbohydrate-anchoring asparagine to an aspartic acid (22, 23). SDS-PAGE analysis and autoradiography were used to monitor the degree of deglycosylation attained (Fig. 4, *A* and *C*), and the resulting preparations were used in a solution binding assay to measure their ternary complex forming ability (Fig. 4, *B* and *D*). Fig. 4*A* depicts the SDS-PAGE analysis of [^{125}I]ALS treated under conditions that fully removed *N*-linked sugars. The binding curves (Fig. 4*B*) reveal that there is no specific binding of either the IGF-I·IGFBP-3 or the IGF-II·IGFBP-3 binary complex with the fully deglycosylated ALS preparation. Therefore, the complete removal of *N*-linked sugars from ALS by Endo F abolishes ternary complex formation.

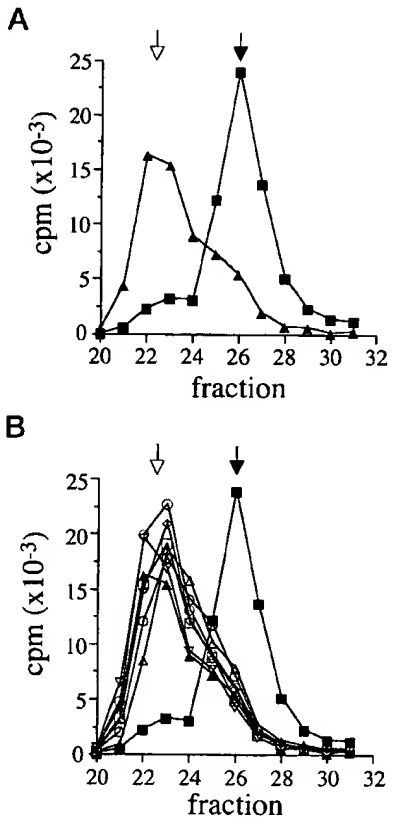

FIG. 5. **ALS mutants lacking individual glycan attachment sites bind to IGFBP-3.** *Panel A*, cross-linked [^{125}I]IGF-I·IGFBP-3 was incubated with media conditioned by CHO cells transfected with pMSG (■) or 150 ng of recombinant wild type ALS (▲) in a volume of 200 μl and then analyzed by size fractionation on a Superose-12 column. The *open* and *closed arrows* represent ~140- and ~50-kDa complexes, respectively. *Panel B*, in a similar series of experiments cross-linked [^{125}I]IGF-I·IGFBP-3 was incubated with media containing 150 ng of recombinant mutant ALS species lacking specific glycan attachment sites (△, Asn^{37} → Ala; ◇, Asn^{58} → Ala; □, Asn^{69} → Ala; ○, Asn^{341} → Ala; ▽, Asn^{527} → Ala; ⊙, Asn^{553} → Ala), and resulting complexes were analyzed on a Superose-12 column. In a separate experiment, recombinant ALS mutant Asn^{448} → Ala displayed a similar increase in size when incubated with [^{125}I]IGF-I·IGFBP-3.

We also generated a series of partially deglycosylated [^{125}I]ALS preparations (Fig. 4*C*). As seen in the binding curves depicted in Fig. 4*D*, the most effectively deglycosylated preparations were the least able to form complexes with IGF-I and IGFBP-3, whereas the preparation with almost fully intact *N*-glycosylation was virtually indistinguishable from the control. Therefore, although the complete removal of *N*-linked sugars from ALS abolishes ternary complex formation, ALS with some intact *N*-linked sugars is able to form the complex.

No N-Linked Sugar Is Solely Responsible for ALS Binding Activity—The binding ability of a protein can be substantially altered by the removal of a single *N*-linked carbohydrated chain (24). Since we demonstrated that the *N*-linked sugars on ALS have a role in ternary complex formation, we used site-directed mutagenesis to investigate whether any single glycan chain was solely responsible for the binding of ALS to the IGF

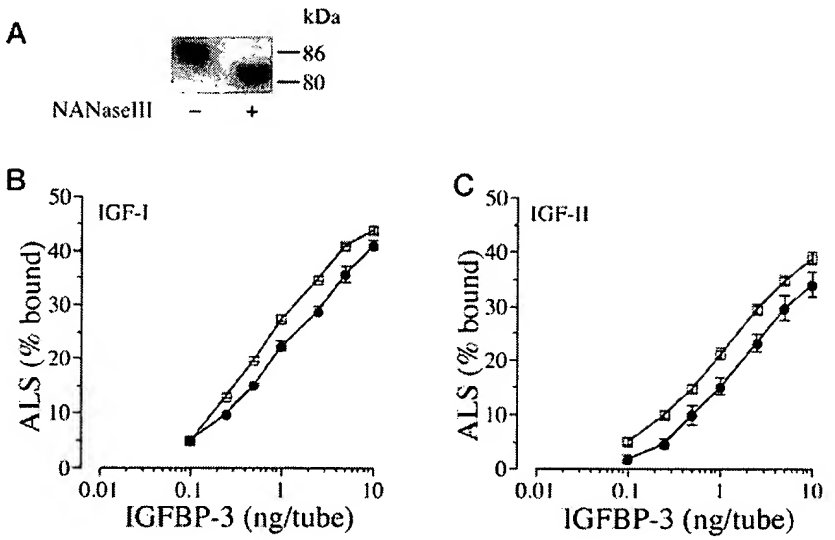

FIG. 6. **NANase III-treated ALS has a reduced ability to bind IGFBP-3.** Preparations of [^{125}I]ALS were treated with or without NANase III overnight at 37 °C and then subjected to nonreducing SDS-PAGE; a representative autoradiograph is shown in *panel A*. The untreated (□) and NANase III-treated (●) [^{125}I]ALS were then assayed for binding to increasing amounts of IGFBP-3 in the presence of 10 ng of IGF-I (*panel B*) and IGF-II (*panel C*). Desialylation of the [^{125}I]ALS used in these experiments was confirmed by SDS-PAGE (see *panel A*). The binding curves represent means ± S.E. of three separate experiments.

and IGFBP-3 complex. The primary sequence of ALS has seven consensus NXS/T sites for *N*-linked sugar attachment. Therefore, we constructed a series of seven mutant ALS cDNAs. The mutations were at $Asn^{37} \rightarrow Ala$, $Asn^{58} \rightarrow Ala$, $Asn^{69} \rightarrow Ala$, $Asn^{341} \rightarrow Ala$, $Asn^{448} \rightarrow Ala$, $Asn^{527} \rightarrow Ala$ and $Asn^{553} \rightarrow Ala$. The mutant ALS cDNAs were then transfected into CHO cells, and the proteins were harvested. All transfections resulted in conditioned medium containing measurable amounts of immunoreactive ALS, as determined by radioimmunoassay (17). From this, we concluded that no single *N*-linked sugar is an absolute requirement for secretion of ALS by CHO cells.

The conditioned media from the transfectants were then used to determine the ternary complex forming ability of the various glycosylation mutant ALS proteins. Ternary complex formation was evaluated by size shift of a cross-linked [^{125}I]IGF-I·IGFBP-3 complex on a Superose-12 gel permeation column (Fig. 5). The peak radioactivity found in fraction 26 with the pMSG control (Fig. 5*A*) is consistent with the size of the IGF-I·IGFBP-3 binary complex (18). In contrast, media from wild-type ALS transfected CHO cells caused a clear shift in the peak fraction of radioactivity from fraction 26 to fraction 23 (Fig. 5*A*). Similar chromatography profiles were obtained for all the mutant ALS forms and are depicted in Fig. 5*B*, except $Asn^{448} \rightarrow Ala$, which gave similar results in a separate assay (data not shown). In each case, the shift in peak radioactivity from fraction 26 to fraction 23 is consistent with the formation of ternary complexes. Therefore, we conclude that all of the mutant ALS proteins are able to bind to the IGF-I·IGFBP-3 complex. Hence, no single *N*-linked sugar of ALS can solely account for the loss of activity observed in the enzymatic deglycosylation experiments.

NANase III-treated ALS Has a Reduced Affinity for IGFBP-3—Having established that ALS glycosylation has a role in ternary complex formation, we investigated whether negatively charged sialic acid moieties may be specifically involved in ALS binding to the IGF·IGFBP-3 complex. Binding curves for the interaction of NANase III-treated ALS and untreated ALS with IGFBP-3 are depicted in Fig. 6. In the presence of IGF-I, desialylation of ALS significantly shifted the binding curve to the right ($p = 0.02$) (Fig. 6*B*), indicative of a decrease in ALS binding. A similar result was obtained in the presence of IGF-II ($p = 0.005$) (Fig. 6*C*). Therefore, ternary complex formation in the presence of either IGF-I or IGF-II is reduced after enzymatic desialylation of ALS. One of the desialylated preparations used in this study was also used in the IEF

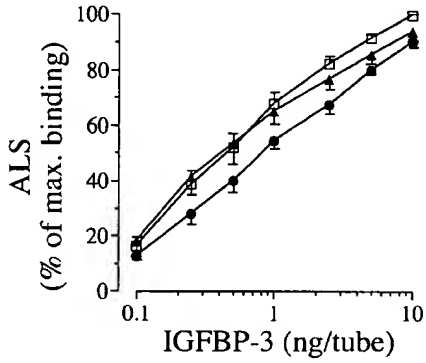

FIG. 7. **Binding of ALS to IGFBP-3 is specifically dependent on sialylation.** Preparations of [^{125}I]ALS either treated with NANase III (●) or with NANase III in the presence of the sialic acid analog DANA (1 nmol/milliunit NANase III, ▲) or with no treatment (□) were incubated for up to 16 h at 37 °C (see "Experimental Procedures"). These preparations were then assayed for binding to increasing amounts of IGFBP-3 in the presence of 10 ng of IGF-I. All complex formation curves were corrected for nonspecific binding and then expressed as the percentage of maximum binding of the control (untreated [^{125}I]ALS in the presence of 10 ng of IGFBP-3 and 10 ng of IGF-I).

depicted in Fig. 3, demonstrating an increase in pI compared with control [^{125}I]ALS and demonstrating that the preparation was fully desialylated under the conditions used.

The specificity of NANase III treatment was tested by blocking its sialidase activity with the sialic acid analog DANA. ALS treated with NANase III in the presence of DANA showed IGFBP-3 binding activity that was not significantly different from the untreated protein (Fig. 7). This suggested that NANase III specifically removed the sialic acid moieties from ALS and that these sialic acid moieties were necessary for normal ternary complex formation.

Finally, Scatchard analysis was used to determine the difference in affinities of NANase III-treated ALS and untreated ALS for IGF-I·IGFBP-3 and IGF-II·IGFBP-3 complexes. Representative Scatchard plots for IGF-I ternary complex formation are shown in Fig. 8, indicating a decrease in affinity of ALS for IGFBP-3 after desialylation. Representative association constants derived from two experiments are shown in Table I. These experiments show that desialylated ALS has a 50–80% reduction in affinity for IGFBP-3 in the presence of IGF-I or IGF-II compared with normally sialylated ALS.

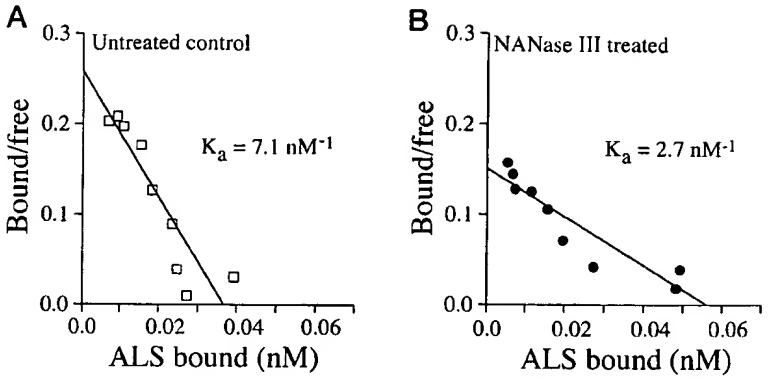

FIG. 8. **Reduced affinity of desialylated ALS for ternary complex formation.** Representative Scatchard analyses comparing the affinities of untreated (*panel A*) and NANase III-treated (*panel B*) ALS for 1 ng of IGFBP-3 in the presence of 10 ng of IGF-I.

TABLE I
Summary of affinities of untreated and NANase III-treated ALS for IGF·IGFBP-3 complexes

The affinities of untreated and desialylated ALS for both IGF-I·IGFBP-3 and IGF-II·IGFBP-3 complexes were determined by Scatchard analyses. The results are derived from two separate experiments.

IGF	K_a	
	Untreated	NANase III-treated
	nM^{-1}	
IGF-I	7.4	3.4
IGF-I	7.1	2.7
IGF-II	4.6	2.3
IGF-II	5.8	1.1

DISCUSSION

The interaction of ALS with IGF·IGFBP-3 complexes in the serum is believed to regulate both the function and the stability of the bound IGFs and IGFBP-3. Previous reports indicate that the affinity of IGFBP-3 for its ligands may be affected by post-translational modifications such as limited proteolysis (25–27). In this study, we found that the carbohydrate chains on ALS play an influential role in determining its interaction with IGFBP-3. Our data therefore suggest that modifications to ALS as well as IGFBP-3 may be important in fine-tuning the bioavailability of the IGFs.

In addition to the seven putative *N*-linked carbohydrate attachment sites (N*X*S/T) in the ALS sequence, there are two putative *O*-linked glycan sites. The first, at Ser^{60}, conforms to a mucin-type *O*-glycosylation site as predicted by NetOglyc 2.0 (28) and is part of the *N*-linked carbohydrate attachment site at Asn^{58}. The second, $FT^{494}PQP$, corresponds to the *X*TP*X*P sequence recently described as the minimal requirement for *O*-linked carbohydrates (29) and lies adjacent to the putative *N*-linked glycosylation site, Asn^{448}. Treatment of ALS with *O*-glycosidase, which hydrolyzes Galβ(1–3)GalNAc, a common core structure of many *O*-linked glycans (30), had no effect on the molecular mass of ALS as judged by SDS-PAGE analysis. While it is possible that fucose residues may have interfered with the enzyme, or that a shift in size was below the level of detection, the putative *O*-linked sites do not appear to be occupied by sugar side chains with the common core structure described above. Furthermore, a lectin binding assay that was specific for sialic acids, a common residue on *O*-linked carbohydrates, also failed to provide evidence for *O*-linked sugars on ALS.

Therefore, we focused on the *N*-linked carbohydrates of ALS and their role in ternary complex formation. Enzymatic removal of the *N*-linked sugars from ALS decreased its ability to form the complex with IGFBP-3 in a manner that was related to the level of deglycosylation. Intermediate levels of ALS deglycosylation, probably involving the loss of more than a single sugar chain, measurably disrupted ternary complex formation, whereas complete removal of the *N*-linked glycans abolished complex formation. However, when site-directed mutagenesis was used to mutate each of the *N*-linked attachment sites individually, no single glycan appeared to have a major impact on ALS binding activity. Therefore, the carbohydrates on ALS clearly influence the affinity that ALS has for the IGF·IGFBP-3 complex, although this influence relies on a number of *N*-linked sugars rather than any single chain. However, it is not clear whether deglycosylated ALS is less able to form the ternary complex because of conformational changes in ALS induced by removal of the glycans or disruption of interactions between the ALS carbohydrates and the other two proteins.

Sialic acids are common anionic residues that can be attached to both *N*- and *O*-linked sugars. They can contribute significant charge to glycoproteins as many sialic acids can be attached to one highly branched *N*-linked sugar. For example, the acute-phase protein α_1-acid glycoprotein has a pI of 2–3 mostly due to the large number of sialic acids attached to its highly branched complex *N*-linked sugars (31). The calculated pI of ALS, based on its amino acid sequence, is 6.56; however, we observed six discrete isoforms of pI 4.6–5.3 by isoelectric focusing. After NANase III treatment, the pI of ALS increased to between 5.5 and 7, close to the predicted value for the amino acid backbone. The contribution of negative charge by the sialic acid may explain, in part, the high affinity with which ALS binds to weak anion exchange columns used in ALS purification (3). Given that the sialic acid contributes significantly to the negative charge on ALS and that the interactions within the ternary complex are dependent on charge, it could be predicted that the sialic acid on ALS would affect the formation of the complex. Indeed, ALS treated with NANase III to remove sialic acid displayed a 50–80% decrease in affinity for the IGF-I and IGF-II binary complexes compared with that of untreated ALS. However, it is noteworthy that desialylation only lowered the affinity of ALS for the IGF·IGFBP-3 complex unlike deglycosylation, which abolished complex formation. This suggests that the effects of ALS glycosylation on IGFBP-3 binding are not entirely due to the negative charges imparted by sialic acid.

The results from the *N*-linked glycan studies imply that a number of *N*-linked sugars are required to act in concert to enable ALS to interact with IGFBP-3 and the IGFs, rather than a specific carbohydrate chain being solely responsible. The placement of the glycans within the tertiary structure of ALS may shed light on this finding. In unpublished studies,[2] we have modeled the central leucine-rich repeat region of ALS on the only published crystal structure of a leucine-rich repeat protein, the porcine ribonuclease inhibitor (32). If the modeled

[2] J. B. M. Janosi, P. A. Ramsland, M. Mott, S. M. Firth, R. C. Baxter, and P. J. D. Delhanty, unpublished data.

structure is a true representation of ALS, then six out of the seven potential *N*-linked sugar attachment sites lie very close to each other, suggesting a possible clustering of carbohydrate chains. Therefore, the loss of any single *N*-linked glycan may be compensated by the potentially large number of other carbohydrates in the vicinity. This model also has implications with regard to the sialic acid moieties that we demonstrated to exist on ALS. A lectin solution binding assay suggested that all the sialic acids on ALS are attached to the *N*-linked sugars. These sialic acid moieties might therefore result in a region of negative charge where the *N*-linked sugars are concentrated.

The binding affinity of ALS to the IGF·IGFBP-3 complex is relatively weak, 1–2 orders of magnitude less than the affinity of IGFBP-3 for either of the IGFs (13), and in this sense ALS binding is the limiting step in ternary complex formation. This suggests that modulation of ALS affinity might directly influence complex formation, and thus the bioavailability of IGFs. Isoelectric focusing indicates that ALS purified from normal serum already exists as a number of differently sialylated isoforms. If the degree of ALS sialylation is subject to physiological regulation, as described for other proteins (31), the potential exists for modulation of formation or stability of the ternary complexes. This might occur in addition to the previously described modulation of the circulating concentration of the ALS protein itself (33) through cytokine suppression (34) or in patients who are critically ill (35) or have hepatic cirrhosis (36).

In summary, we have found that the *N*-linked carbohydrates on ALS are a requirement for the formation of complexes with IGFBP-3 and IGFs. We have also shown that the removal of sialic acids from ALS significantly reduces the affinity of ALS for binding to IGFBP-3. Since the glycosylation of secreted proteins are often modified in certain physiological and pathological states, the modification of ALS glycosylation has the potential to be an important factor in the regulation of IGF access to the tissues.

REFERENCES

1. Jones, J. I., and Clemmons, D. R. (1995) *Endocr. Rev.* **16,** 3–34
2. Rajaram, S., Baylink, D. J., and Mohan, S. (1997) *Endocr. Rev.* **18,** 801–831
3. Baxter, R. C., Martin, J. L., and Beniac, V. A. (1989) *J. Biol. Chem.* **264,** 11843–11848
4. Twigg, S. M., and Baxter, R. C. (1998) *J. Biol. Chem.* **273,** 6074–6079
5. Guler, H. P., Zapf, J., Schmid, C., and Froesch, E. R. (1989) *Acta Endocrinol.* **121,** 753–8
6. Lewitt, M. S., Saunders, H., and Baxter, R. C. (1993) *Endocrinology* **133,** 1797–1802
7. Leong, S. R., Baxter, R. C., Camerato, T., Dai, J., and Wood, W. I. (1992) *Mol. Endocrinol.* **6,** 870–876
8. Kobe, B., and Deisenhofer, J. (1995) *Curr. Opin. Struct. Biol.* **5,** 409–416
9. Dai, J., and Baxter, R. C. (1992) *Biochem. Biophys. Res. Commun.* **188,** 304–309
10. Delhanty, P., and Baxter, R. C. (1996) *Biochem. Biophys. Res. Commun.* **227,** 897–902
11. Boisclair, Y. R., Seto, D., Hsieh, S., Hurst, K. R., and Ooi, G. T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10028–10033
12. Liu, F., Hintz, R. L., Khare, A., Diaugustine, R. P., Powell, D. R., and Lee, P. D. (1994) *J. Clin. Endocrinol. Metab.* **79,** 1883–1886
13. Holman, S. R., and Baxter, R. C. (1996) *Growth Regul.* **6,** 42–47
14. Baxter, R. C. (1990) *Biochem. J.* **271,** 773–7
15. Firth, S. M., Ganeshprasad, U., and Baxter, R. C. (1998) *J. Biol. Chem.* **273,** 2631–2638
16. Baxter, R. C., Bayne, M. L., and Cascieri, M. A. (1992) *J. Biol. Chem.* **267,** 60–65
17. Baxter, R. C. (1990) *J. Clin. Endocrinol. Metab.* **70,** 1347–1353
18. Baxter, R. C. (1988) *J. Clin. Endocrinol. Metab.* **67,** 265–272
19. Chaney, W. G., Howard, D. R., Pollard, J. W., Sallustio, S., and Stanley, P. (1986) *Somat. Cell Mol. Genet.* **12,** 237–244
20. Abidi, F. E., Bishayee, S., Bachhawat, B. K., and Bhadra, R. (1987) *Anal. Biochem.* **166,** 257–66
21. Dorai, D. T., Bachhawat, B. K., and Bishayee, S. (1981) *Anal. Biochem.* **115,** 130–7
22. Tarentino, A. L., Gomez, C. M., and Plummer, T. J. (1985) *Biochemistry* **24,** 4665–71
23. Maley, F., Trimble, R. B., Tarentino, A. L., and Plummer, T. J. (1989) *Anal. Biochem.* **180,** 195–204
24. Avvakumov, G. V., Warmels-Rodenhiser, S., and Hammond, G. L. (1993) *J. Biol. Chem.* **268,** 862–866
25. Baxter, R. C., and Skriver, L. (1993) *Biochem. Biophys. Res. Commun.* **196,** 1267–1273
26. Bang, P., Brismar, K., and Rosenfeld, R. G. (1994) *J. Clin. Endocrinol. Metab.* **78,** 1119–1127
27. Baxter, R. C., Suikkari, A. M., and Martin, J. L. (1993) *Biochem. J.* **294,** 847–852
28. Hansen, J. E., Lund, O., Tolstrup, N., Gooley, A. A., Williams, K. L., and S., B. (1998) *Glycoconj. J.* **15,** 115–130
29. Yoshida, A., Suzuki, M., Ikenaga, H., and Takeuchi, M. (1997) *J. Biol. Chem.* **272,** 16884–16888
30. Umemoto, J., Bhavanandan, V. P., and Davidson, E. A. (1977) *J. Biol. Chem.* **252,** 8609–8614
31. Van Dijk, W., Havenaar, E. C., and Brinkman-van der Linden, E. C. M. (1995) *Glycoconj. J.* **12,** 227–33
32. Kobe, B., and Deisenhofer, J. (1993) *Nature* **366,** 751–756
33. Delhanty, P. J. D. (1998) *The Regulation and Actions of ALS: Molecular Mechanisms to Regulate the Activities of Insulin-like Growth Factors* (Takano, K., Hizuka, N., and Takahashi, S. I., eds) pp. 135–143, Elsevier Science B. V., Amsterdam
34. Delhanty, P. J. D. (1998) *Biochem. Biophys. Res. Commun.* **243,** 269–272
35. Baxter, R. C., Hawker, F. H., To, C., Stewart, P. M., and Holman, S. R. (1997) *Growth Regul.* **7,** 1–10
36. Donaghy, A. J., and Baxter, R. C. (1996) *Baillieres Clin. Endocrinol. Metab.* **10,** 421–446